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PRINCIPAL INVESTIGATOR: Zafar Nawaz, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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6. AUTHOR(S)
Zafar Nawaz, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-Mail: zn029815@bcm.tmc.edu

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13. Abstract ***(Maximum 200 Words)*** Steroid hormones, estrogen and progesterone, and their intracellular receptors play an important role in the development and progression of breast cancer. Coactivator proteins modulate the biological activity of these hormone receptors. We have cloned an E3 ubiquitin-protein ligase enzyme, E6-associated protein (E6-AP) and E2 ubiquitin-conjugating enzyme, UbcH7 as coactivators of steroid hormone receptors. The purpose of this research is to explore the possibility that the altered expression of E6-AP and UbcH7 may contribute to the development of breast cancer. We have examined this possibility by studying the expression patterns of E6-AP, UbcH7 and estrogen receptor-alpha (ER) in various human breast cancer cell lines and breast tumor biopsy samples. Additionally, we have correlated the expression profile of E6-AP and UbcH7 with that of ER in breast tumor biopsies. Todate, we have examined 56 advanced stage human breast cancer biopsy samples for the expression profile of E6-AP, UbcH7 and ER. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis. However, we did not find any statistically significant correlation between the expression profile of UbcH7 and ER in these tumor samples. Presently, we are studying the expression profile of E6-AP, UbcH7 and ER in early and intermediate stage tumors. Another goal of this project is to create novel in vitro models in stable cell lines, which will overexpress coactivator proteins, E6-AP and UbcH7. In order to achieve this goal, we have already constructed the expression vectors for stable cell lines. Our preliminary data suggest that these vectors produce biologically functional coactivator proteins, E6-AP and UbcH7. Presently, we are in the process of generating the stable cell lines that will stably overexpress E6-AP and UbcH7.

14. SUBJECT TERMS
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Introduction

Breast cancer is the leading cause of death in American women. It is anticipated that one woman out of ten will develop breast cancer at some point during her life (Nicholson 1979; Nicholson et al. 1986; Horwitz 1994; Nicholson et al. 1995; Parker et al. 1997; Morris et al. 2001). Although in recent years significant progress has been made in detection and treatment of the disease, much of the molecular basis of the disease remains unknown. This fact highlights the need to identify and understand the molecular basis associated with breast cancer development and progression.

Steroid hormones, estrogen and progesterone, play important role in the development and progression of breast cancer (Benner et al. 1988; Clarke et al. 1989; Clarke et al. 1992; Elledge et al. 2000). Estrogens and progesterones exert their biological effects on target tissues through intracellular receptor proteins, the estrogen (ER) and progesterone (PR) receptors (O'Malley 1990; Tsai and O'Malley 1994; Hatina and Reischig 2000). These receptors contain common structural motifs which include a less well conserved amino-terminal activation function (AF-1) that effects transcription efficiency, which has the hormone-independent activation function; a central DNA-binding domain, which mediates receptor binding to specific DNA enhancer sequences and determine target gene specificity; and a carboxy-terminal hormone-binding domain (HBD). The HBD contains activation function-2 (AF-2); the region mediates the hormone-dependent activation function of the receptors (O'Malley 1990; Tsai and O'Malley 1994; Hatina and Reischig 2000).

In order to activate gene transcription, ER and PR undergo a series of well-defined steps. When bound to hormone, these receptors undergo a conformational change, dissociation from cellular chaperones, receptor dimerization, phosphorylation, interaction with coactivators and recruitment of chromatin modifying enzyme activities such as histone acetyl transferase activity (HAT) and ATPase activity, DNA-binding at an enhancer element of the target gene, and subsequent recruitment of basal transcription factors to form a stable preinitiation complex (PIC) (Horwitz et al. 1996; McKenna et al. 1998; McKenna 1999; Chen 2000). These events are followed by up- or down-regulation of target gene expression.

Coactivators represent a growing class of proteins, which interact with receptors in a ligand-specific manner and serve to enhance their transcriptional activity. Prior to their identification, coactivators were predicted to exist based upon experiments, which showed that different receptors compete for a limiting pool of accessory factors required for optimal transcription. Stimulation of one receptor resulted in trans-repression of another receptor, indicating the depletion of a common coactivator pool (Bocquel et al. 1989; Meyer et al. 1989; Shemshedini et al. 1992). A number of coactivators have been cloned to date, including SRC-1 (Onate et al. 1995), TIF2 (GRIP1) (Hong et al. 1996; Voegel et al. 1996; Hong et al. 1997; Voegel et al. 1998), p/CIP (ACTR/RAC3/AIB1/TRAM-1) (Anzick et al. 1997; Chen et al. 1997; Li et al. 1997; Takeshita et al. 1997; Torchia et al. 1997), PGCs (Puigserver et al. 1998), SRA (Lanz et al. 1999), CBP (Ikonen et al. 1997; Aarnisalo et al. 1998; Fronsdal et al. 1998), **E6-associated protein (E6-AP), and ubiquitin conjugating enzymes such as UbcH5B, UbcH7 and Ubc9** (Nawaz et al. 1999b; Poukka et al. 1999; Poukka et al. 2000) and this list is growing rapidly day by day.

Coactivators were originally envisioned to serve a bridging role, linking the receptor to the basal transcription machinery (Pugh and Tjian 1992; Tjian and Maniatis 1994). Recently, the functional role of coactivators has expanded by the observation that they have been shown to possess enzymatic activities that may contribute to their ability to enhance receptor mediated transcription; SRC-1, p300/CBP, and ACTR (RAC3/AIB1) possess a histone

acetyl transferase, HAT, activity (Ogryzko et al. 1996; Anzick et al. 1997; Li et al. 1997; Spencer et al. 1997; McKenna et al. 1998; Collingwood et al. 1999; Chen 2000) and members of SWI/SNF complex contain an ATPase activity (Dunaief et al. 1994; Muchardt et al. 1996; Wang et al. 1996; Reyes et al. 1997). Ligand-activated receptors are thought to bring HAT and ATPase activities containing coactivators to the chromatin surrounding the receptor, disrupting the local repressive chromatin structure by acetylating histones and possibly other chromatin associated factors and catalyzing the uncoupling of ionic interactions between histones and their substrate DNA (Dunaief et al. 1994; Muchardt et al. 1996; Ogryzko et al. 1996; Wang et al. 1996; Yang et al. 1996; Reyes et al. 1997; Spencer et al. 1997). Because of their ability to enhance receptor mediated gene expression, coactivators are thought to play an important role in regulating the magnitude of the biological responses to hormones (Xu et al. 1998; McKenna 1999; Leo and Chen 2000; Xu et al. 2000). The level of coactivator expression is critical in determining the activity of the receptor in target tissues and variations in hormone responsiveness seen in the population may be due to differences in coactivator levels.

It is accepted that coactivators either possess or bring HAT and ATPase activities to the promoter region of the target genes and presumably manifest part of their in vivo coactivation functions through these enzymatic activities (Dunaief et al. 1994; Muchardt et al. 1996; Ogryzko et al. 1996; Wang et al. 1996; Reyes et al. 1997; Spencer et al. 1997). Recent identification of the enzymes of the ubiquitin-proteasome and ubiquitin-like pathways as coactivators by my own laboratory and others added a new twist to the coactivator field. These studies suggest that the ubiquitin-conjugating enzymes, UbcH5B, UbcH7 and Ubc9 and the E3 ubiquitin-protein ligases, E6-AP and RPF1/RSP5, interact with members of the steroid hormone receptor superfamily including ER and PR and modulate their transactivation functions (Imhof and McDonnell 1996; McKenna et al. 1998; Nawaz et al. 1999b; Poukka et al. 1999; Poukka et al. 2000). Similarly, another coactivator protein, yeast SUG1, an ATPase subunit of the 26S-proteasome complex also interacts with and modulates steroid hormone receptor function (Fraser et al. 1997; Makino et al. 1997; Masuyama and MacDonald 1998). Instead of HAT activity, this group of coactivators possesses other enzymatic activities such as ubiquitin conjugation, ubiquitin ligation and protease activities. However, a common theme between the two groups of coactivators is that both possess some sort of enzymatic activity.

As mentioned above, my laboratory has identified ubiquitin pathway enzymes as coactivators of the nuclear hormone receptor superfamily. We have cloned an E3 ubiquitin-protein ligase, E6-AP as steroid hormone receptor interacting protein using a yeast two-hybrid screening assay. E6-AP enhances the hormone-dependent transcriptional activity of steroid hormone receptors, PR, ER, androgen (AR) and glucocorticoid receptors (GR) (Nawaz et al. 1999b). E6-AP was previously identified as a protein of 100 kDa, present both in the cytoplasm and the nucleus. E6-AP mediates the interaction of human papillomaviruses type 16 and 18 E6 proteins with p53, a growth-suppressive and tumor-suppressive protein. The E6/E6-AP complex specifically interacts with p53 and promotes the degradation of p53 via the ubiquitin-proteasome protein degradation pathway (Huibregtse et al. 1991; Huibregtse et al. 1993). As mentioned above, E6-AP is a member of the E3 class of functionally related ubiquitin-protein ligases. E3 enzymes have been proposed to play a major role in defining substrate specificity of the ubiquitin system (Scheffner et al. 1993; Huibregtse et al. 1995b; Huibregtse et al. 1995a). Protein ubiquitination also involves two other classes of enzymes, namely the E1 ubiquitin activating enzyme (UBA) and many E2 ubiquitin conjugating enzymes (UBCs). The UBA first activates ubiquitin in an ATP-dependent manner. The activated ubiquitin then forms a thioester bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of the UBA. Next, ubiquitin is transferred from the E1 to one of the several E2s (UBCs), preserving the high-energy thioester bond. In some cases, ubiquitin is transferred

directly from the E2 to the target protein through an isopeptide bond between the ε-amino group of lysine residues of the target protein and the carboxyl-terminus of ubiquitin. In other instances, the transfer of ubiquitin from UBCs to target proteins proceeds through an E3 ubiquitin-protein ligase intermediate such as E6-AP (Ciechanover 1994; Ciechanover and Schwartz 1994).

The E2 ubiquitin conjugating enzymes of the ubiquitin pathway, UbcH5B and UbcH7 (UBCs) also act as coactivators of steroid hormone receptors. Furthermore, we have also demonstrated that the ER protein, which is a major modulator of normal mammary gland development and breast tumor development, is rapidly degraded in mammalian cells in an estrogen-dependent manner. Treatment of mammalian cells with the proteasome inhibitor, MG132, which specifically blocks the protease activity of the proteasome, blocks ER degradation. This suggests that ER protein is degraded through the ubiquitin-proteasome pathway (Nawaz et al. 1999a). In addition, our results also suggest that the estrogen-dependent degradation of ER correlates with hormone-dependent ER activation because MG132 not only blocks ER protein degradation but also block its activation. Our *in vitro* studies suggest that ER degradation observed in mammalian cells is dependent on the UBCs, UbcH5B and UbcH7 and ubiquitin-proteasome pathway (Nawaz et al. 1999a). These observations raise the question as to why ubiquitin pathway enzymes and ubiquitin-dependent protein degradation are linked to steroid hormone receptor activation. Considering that the transcriptionally active receptor is associated with a diverse group of proteins and forms a preinitiation complex, it is possible that subsequent to receptor activation of transcription, ubiquitin mediated degradation of the receptor may be a mechanism which dissociates the preinitiation complex. It could be necessary to dissociate the preinitiation complex through targeted protein degradation since the synergistic interactions of multiple transcription factors may make passive dissociation of hormone and coactivators impossible or time consuming. Additionally, it is possible that hormone-induced receptor degradation serves to control physiological responses to steroid hormones ultimately limiting the expression of steroid-responsive genes.

It has been shown that altered expression of one nuclear receptor coactivator, AIB1, contributes to the development of hormone-dependent breast and ovarian cancer. Interaction of AIB1, SRC-1, TIF2, and p/CIP with CBP/ p300 is important for the coactivation function. Thus, overexpression or loss of expression of any of these coactivators could potentially perturb signal integration by CBP/ p300 and affect multiple transduction pathways (Anzick et al. 1997). Recenly, it has also been shown that another steroid receptor coactivator, SRA, is also elevated in breast tumors (Murphy et al. 2000). Furthermore, we have also shown that E6-AP is overexpressed 2.5-4.5 fold in 90-95% of tumors using a mouse mammary model of multistage tumorigenesis. E6-AP is overexpressed only in tumors but not in the intermediate steps of tumorigenesis (Sivaraman et al. 2000).

The purpose of this research is to explore the possibility that the altered expression of UbcH5B, UbcH7 and E6-AP may contribute to the development of breast cancer. In the original proposal, we proposed to explore this possibility by studying the expression profile of UbcH5B, UbcH7, and ER in various breast cancer cell lines and breast tumor biopsy samples. We also proposed to create novel in vitro models in stable cell lines, which will overexpress coactivator proteins, UbcH5B and UbcH7. We have examined the expression patterns of E6-AP, UbcH7, with that of ER in various human breast cancer cell lines and breast tumor biopsy samples. Additionally, we have also correlated the expression profile of E6-AP and UbcH7 with that of ER in breast tumor biopsies. Unfortunately, due to the lack of good UbcH5B antibody, we are unable to study the expression profile of UbcH5B. Instead of UbcH5B, we have examined the expression profile of E6-AP in several breast

cancer cell lines and breast biopsy tumors. By Western blot analysis, we have examined 56 advanced stage human breast cancer biopsy samples for the expression profile of E6-AP, UbcH7 and ER; and by immunohistochemistry, we have compared the expression profiles of E6-AP in 12 breast tumors and their adjacent normal tissues. We have also compared the expression profiles of E6-AP in different stages of tumors by dual immunofluorescence (36 tumors). Additionally, we have also compared the expression profiles of E6-AP with that of ERα in 27 human breast biopsy samples and their adjacent normal tissues. Our data demonstrate an inverse correlation between the expression of E6-AP and the expression of ERα in these tumors both by Western blot and by immunofluorescent analysis. In both cases, the Spearman Rank Correlation Coefficient is below 0.05, $p< 0.05$, indicating that this correlation is statistically significant. Additionally, we also found that the expression of E6-AP in tumors is lower compared with their adjacent normal tissues, while this decrease is often accompanied by the increase of ERα expression. We also found that the decrease in expression of E6-AP is stage-dependent, with stage IIB being the lowest point. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis. However, we did not find any statistically significant correlation between the expression profile of UbcH7 and ER in these tumor samples. Another goal of this project is to create novel in vitro models in stable cell lines, which will overexpress coactivator proteins, E6-AP and UbcH7. In order to achieve this goal, we have constructed the expression vectors for stable cell lines using pcDNA3.1. However, our attempt to stably express these proteins in MCF-7 cell lines was not successful due to constitutive high expression of E6-AP and UbcH7. To circumvent this problem we are currently constructing inducible expression vectors where we can iduce the expression of E6-AP and UbcH7. Due to the flooding caused by the tropical Allison in June of 2001, we lost all the tumor samples and cell lines we had and we had to start all over again, which greatly hampered the progress of this project.

Body

In this original proposal, we hypothesized that ubiquitin-conjugating enzymes, UbcH5B, UbcH7 and an E3 ubiquitin-protein ligase, E6-AP, are important modulators of the steroid hormone receptor-mediated signal transduction pathway, cell growth, and cell cycle control in the context of breast cancer development. In order to test this hypothesis we propose following objectives:

- **Expression analysis of endogenous ubiquitin-conjugating enzymes, UbcH5B and UbcH7, and ER in breast cancer cell lines and human breast tumor biopsies. Then compare the expression patterns of UbcH5B and UbcH7 with that of ER.**
- **Design and development of stable in vitro models of UbcH5B and UbcH7 overexpression in the breast cancer cell lines.**
- **Analysis of the growth properties of stably transfected cell lines that overexpress UbcH5B and UbcH7 and in vivo analysis of tumorigenicity of these stably transfected cell lines in athymic nude mice.**

Expression analysis of endogenous ubiquitin-conjugating enzymes, UbcH5B and UbcH7, and ER in breast cancer cell lines and human breast tumor biopsies. Then compare the expression patterns of UbcH5B and UbcH7 with that of ER.

One of the aims of this proposal is to test the expression of endogenous UbcH5B, UbcH7 and ER in human breast cancer cell lines and human breast tumor biopsies. Then compare the expression profile of UbcH5B and UbcH7 with that of ER. To date we have examined expression levels of UbcH7, E6-AP, and ER in 56 different breast tumors and expression of

p53 in 20 different tumors by Western blot analysis. By immunohistochemistry, we have compared the expression profiles of E6-AP in 12 breast tumors and their adjacent normal tissues and we have also compared the expression profiles of E6-AP in 36 tumors that are at different stages. By dual immunofluorescence, we have also compared the expression of E6-AP with that of ER in 27 different breast tumor samples. Additionally, we have also examined the expression profile of UbcH7, E6-AP and ER in different breast cancer cell lines. Due to the lack of the availability of the UbcH5B antibody, we are unable to examine the expression profile of UbcH5B. Furthermore, we were not successful in generating a good UbcH5B antibody. Since these proteins are also targets of the ubiquitin-proteasome pathway, we did not analyze the mRNA levels of UbcH5B. We found (1) an inverse correlation between the expression of E6-AP and the expression of ER in human biopsy tumor samples; (2) we found that the expression of E6-AP in tumors is lower compared with their adjacent normal tissues; furthermore, this decrease is often accompanied by the increase of ERα expression, and (3) we found that the expression of E6-AP is stage-dependent, with stage IIB expressing lowest levels of E6-AP. However, we did not find any statistically significant correlation between the expression profile of UbcH7 and ER and we did not find any significant changes of UbcH7 expression levels in tumors

Task 1. Expression analysis of UbcH7, ER and E6-AP in different breast cancer cell lines.

A. UbcH7 expression

We have analyzed the expression profile of UbcH7, ER and E6-AP in different breast cancer cell lines such as MCF-7, T47-D, ZR75-1 and MDA-MB-231. As a control we have also examined the expression profile of UbcH7, ER and E6-AP in HeLa (a cervical carcinoma cell line) cells. As shown in Figure 1, HeLa cells express high levels of UbcH7 protein compare to that of different breast cancer cell lines (MCF-7, T47-D, ZR75-1 and MDA-MB-231). Furthermore, in HeLa cells, the UbcH7 expression is both cytoplasmic and nuclear. The expression level of UbcH7 in MCF-7, T47-D, ZR75-1 and MDA-MB-231 is moderate compare to that of HeLa cells. In T47-D cells, the expression of UbcH7 is totally nuclear whereas in other breast cancer cell lines, MCF-7, ZR75-1 and MDA-MB-231, weak cytoplasmic staining of UbcH7 was observed, in addition to nuclear staining.

B. ER expression

Since we want to compare the expression profile of UbcH7 with that of ER, we also analyzed the expression of ER-alpha in MCF-7, T47-D, ZR75-1 and MDA-MB-231 cell lines. In this case, HeLa cell line was used as a negative control. As shown in Figure 2, HeLa cells are negative for ER expression. Similarly, in the breast cancer cell line, MDA-MB-231, the ER expression was undetectable. However, this cell line expresses UbcH7 at moderate level. In contrast to MDA-MB-231, the MCF-7, T47-D and ZR75-1 lines express both UbcH7 and ER-alpha. The ER expression is nuclear in these cell lines.

C. E6-AP expression

Since UbcH7 act as an E2 ubiquitin-conjugating enzyme for E6-AP and both the UbcH7 and E6-AP act as coactivators for ER, we decided to analyze the expression profile of E6-AP in breast cancer cell lines. As shown in Figure 3, the breast cancer cell lines, MCF-7, T47-D, ZR75-1 and MDA-MB-231 express high levels of E6-AP. The E6-AP expression is both cytoplasmic and nuclear in MCF-7, ZR75-1 and MDA-MB-231 cell lines. The MDA-MB-231 cell line expresses more E6-AP in nucleus than in the cytoplasm. Similar to UbcH7, the expression of E6-AP in T47-D cells is mainely nuclear. In this case HeLa cells were used as a positive control for E6-AP expression.

Task 2. Effect of steroids on the expression of UbcH7 and E6-AP.

It is possible that steroid hormones (estrogens/progesterones) may regulate endogenous expression of UbcH7 and E6-AP in breast cancer cell lines. To test this possibility, MCF-7, a hormone-dependent breast cancer cell line was grown in the medium containing stripped serum for a week. Afterward, cells were grown either in the absence or presence of steroid hormones for 48 hours and the expression patterns of UbcH7 and E6-AP were determined by fluorescent immunocytochemistry. As shown in Figure 4, in MCF-7 cells, estrogen treatment has no significant effect on the expression profile of UbcH7. The UbcH7 expression is identical both in the absence and presence of estradiol. Similarly, progesterone treatment also has no significant effect on the expression levels of UbcH7 in MCF-7 cells (data not shown). Next we tested whether steroids treatment has any effect on the expression levels of UbcH7 in T47-D cells. As shown in Figure 5, estrogen treatment has no effect on the expression levels of UbcH7 in T47-D cells. The expression levels of UbcH7 are identical both in hormone treated and untreated cells. The same is true for progesterone (data not shown). These data suggest that the expression of UbcH7 is not under the control of steroid hormones.

Next we ask whether steroids regulate the expression of E6-AP. To test the effect of estrogen on the expression pattern of E6-AP, MCF-7 cells were grown in the medium containing stripped serum for a week. Then, cells were treated with either estrogen or vehicle for 48 hours and the expression patterns of E6-AP was determined by fluorescent immunocytochemistry. Figure 6 suggests that the estrogen treatment have no significant effect on the expression of E6-AP. The E6-AP expression levels are identical both in the presence and absence of hormone. This data suggests that E6-AP regulation is also not under the control of steroids.

As a control for these experiments, we also analyzed the effect of estrogen on the expression of PR and ER. It has been established that estrogen upregulates the expression of PR protein and it down regulates the levels of ER in MCF-7 cells (Lonard et al. 2000). As expected, Figure 7 demonstrate that estrogen treatment increases the expression of PR protein. In contrast, estrogen down regulates ER expression.

Task 3. Expression analysis of ER-alpha, UbcH7 and E6-AP in breast tumor samples.

As mentioned above, the ubiquitin pathway enzymes, UbcH7 and E6-AP act as coactivators of steroid hormone receptors. Furthermore, we have also demonstrated that the ER protein, which is a major modulator of normal mammary gland development and breast tumor development, is rapidly degraded in mammalian cells in an estrogen-dependent manner via the ubiquitin-proteasome pathway. Additionally, our *in vitro* studies suggest that ER degradation observed in mammalian cells is dependent on the UbcH7 and ubiquitin-proteasome pathway (Nawaz et al. 1999a). To explore the possibility that the altered expression of UbcH7 and E6-AP may contribute to the development of breast cancer, we analyzed the expression profile of UbcH7, E6-AP and ER in 56 advanced stage breast tumor biopsy samples by Western blot analysis; we also analyzed 12 pairs of breast tumors with their adjacent normal tissues by Immunohistochemistry. In addition, we have also analyzed 36 samples of different stages of breast tumors by Immunohistochemistry. We also compared the expression of E6-AP with that of ERα in 27 breast tumor samples by Immunofluorescence.

A. Western Blot Analysis

Figure 8 and 12 show the expression profile of ER-alpha in 56 different human tumor samples. 23 (41%) out of 56 breast tumor samples express significant amount of ER-alpha, however, 33 (59%) tumors express no or degraded forms of ER protein. Since we want to

correlate the expression profile of E6-AP and UbcH7 with that of ER in breast tumor biopsies, we examined the expression profile of UbcH7 and E6-AP in these tumors. Figure 9 and 12 show the expression profile of UbcH7 in human breast tumor samples. As shown in Figure 9, majority of the tumors expresses UbcH7. Only 21(%) tumors are negative for UbcH7. Furthermore, we did not find any statistically significant correlation between the expression profile of UbcH7 and ER in these tumor samples.

Since UbcH7 acts as an E2 ubiquitin-conjugating enzyme for E6-AP, we also examined the expression profile of E6-AP in these human breast tumor samples. To study the expression profile of E6-AP in human breast tumors we performed Western blot analysis using an E6-AP specific antibody. Figure 10 shows the expression of E6-AP in 56 different tumor samples. The majority (82%) of the tumors expresses E6-AP. Furthermore, we found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant (Figure 12).

It has been demonstrated that E6-AP promotes the degradation of p53 via the ubiquitin degradation pathway. In the brain of E6-AP knockout animals, the protein levels of p53 accumulate compared to those of normal littermates. Therefore, we also analyzed the endogenous expression of p53 protein from breast tumor biopsies. As shown in Figure 11, p53 expression was not detectable in most tumors except tumor number 7, 10, 13 and 15. Furthermore, there was no statistical correlation between the expression profile of E6-AP and p53.

Since we found an inverse correlation between the expression profile of ER-alpha and E6-AP, we wondered whether E6-AP and ER expression colocalized in breast cancer cell lines and breast cancer tumors. In order to study the colocalization of ER and E6-AP, we performed fluorescent immunocytochemistry on T47-D cells. As shown in Figure 13, the expression of E6-AP colocalized with that of ER (see merge figure). These data suggest that it is possible that in vivo E6-AP may promote the degradation of ER through the ubiquitin-proteasome pathway.

B. Immunohistochemistry

In order to study the expression profile of E6-AP and UbcH7 in breast tumors and in normal breast tissues, we performed immunohistochemical analysis. As shown in Figure 15, in normal human breast tissues, E6-AP is highly expressed in the cytoplasm of the ductal epithelial cells. In contrast, the expression level of E6-AP is much lower in breast tumor tissues. Altogether, we analyzed 12 tumors with their adjacent normal tissues. There are four kinds of outcomes comparing the expression of E6-AP in tumor and in normal tissues: equally high in both tumor and normal tissues; equally low in both cases; high in tumor and low in normal tissues; low in tumor and high in normal tissues. The result is summarized in Figure 16. It is noticed that the majority of the samples (8 out of 12) express lower levels of E6-AP in tumors in comparison to their respective adjacent normal tissues. Chi-Square test shows that the differences between the four groups are statistically significant ($p<0.025$).

Since immunohistochemistry showed that E6-AP is down regulated in breast cancer tissues, and it seems to be in consistent with the results obtained from Western blot analysis and the results from animal studies, we next examined if this change of expression is dependent on the stages or the grades of the tumors. A tumor tissue array with 36 human breast cancer samples in one slide was analyzed by Immunohistochemistry using an antibody against E6-AP. The levels of E6-AP expression were artificially graded according to the intensity of the brown color spots in each sample. Normal tissues was included in the experiment as a

positive control because of their high level expression of E6-AP. Figure 17 shows the distribution of E6-AP expression levels in different stages of human brest tumors. Simple calculation of the means of the levels of expression "X" shows that E6-AP is gradually down regulated from stage I to Stage IIB, then it goes up again. Figure 18 shows the statistical analysis result by comparing the differences between different stages. Stage I and Stage IIIA are significantly different from stage IIB, indicating that stage IIB is probably the lowest point in the E6-AP expression. Additionally, we compared the expression of E6-AP in different grades of breast cancer samples, we could not find any correlation that suggests the change of E6-AP is grade dependent (data not shown).

We also performed immunohistochemical analysis to study the expression of UbcH7 and p53 in breast tumors, but the results are not clear.

C. Immunofluorescence

Combining the data from Western blot and Immunohistochemistry analysis, it is suggested that E6-AP is down regulated in breast tumors and the expression of E6-AP is correlated with that of ER alpha. To confirm this, we further performed Immunohistochemistry to analyze the expression of E6-AP with that of ER. As shown in Figure 19, E6-AP and ERis differently expressed in tumors and in normal tissues: (1) ER is expressed in the nucleus, whereas E6-AP is expressed in the cytoplasm; (2) In normal tissues, ER is discontinuously expressed in the epithelial cells, whereas E6-AP is ubiquitiously expressed in the epithelial cells; (3) In tumor tissues, ER is highly and ubiquitiously expressed in the epithelial cells, whereas the expression of E6-AP is low. Negative control was included in the experiment by omitting the primary antibody. 8 tumor samples together with their respective normal tissues were used in this study. 5 out of 8 tumors that have lower levels of E6-AP expression and higher levels of ER. This result further indicated that the inverse correlation of E6-AP with ER in breast tumors does exist. Additionally, we performed dual immunofluorescence analysis in 19 human breast cancer samples using antibodies aganist E6-AP and ER. Again, the expression levels of E6-AP and ER were artificially graded, which is shown in Figure 20. Wilcoxon Rank Correlation Coefficient is 0.503, $p<0.05$, indicating an inverse correlation between the expression of E6-AP and ER.

Task 4. Generation of the expression plasmids for overexpression of UbcH7 and E6-AP.

To construct the expression plasmids for the overexpression of UbcH7 and E6-AP, cDNAs of UbcH7 and E6-AP were firstly cloned into the mammalian expression vector pcDNA3.1. These vectors produce his-tagged UbcH7 and E6-AP proteins. In order to confirm the functional activity of the his-tagged UbcH7 and E6-AP, we performed the transient transfection assays in HeLa cells. As shown in Figure 14, that the his-tagged UbcH7 and E6-AP were able to enhance the transactivation functions of PR, suggesting that the his-tagged UbcH7 and E6-AP proteins are biologically functional. However, for some reasons, stably transfection of the expression vectors into MCF-7 cells was not successful. Presently, we are constructing new expression vectors, which will express the proteins by an inducible promoter. The constructs have been completed and we are in the process of testing the functions of the vectors by transient transfection

Statement of work accomplished/in progress

Task 1. Expression analysis of UbcH7, ER, and E6-AP in different breast cancer cell lines. **Accomplished.**

Task 2. Effect of steroids on the expression of UbcH7 and E6-AP. **Accomplished.**

Task 3. Expression analysis of ER-alpha, UbcH7, and E6-AP in breast tumor samples. **Accomplished.**

Task 4. Generation of the expression plasmids for overexpression of UbcH7 and E6-AP. **Accomplished.**
Task 5. Development of stable cell lines. **In Progress.**
Task 6. Characterization of stable cell lines. **Not Attempted Yet.**
Task 7. Determination of growth properties of stable cell lines. **Not Attempted Yet.**
Task 8. Determine the tumorigenicity of stably transfected cell lines in athymic nude mice. **Not Attempted Yet.**

Key Research Accomplishments

- Expression analysis of UbcH7, ER, and E6-AP in different breast cancer cell lines has been completed.
- Effect of steroids on the expression of UbcH7 and E6-AP has been studied.
- The expression analysis of ER, UbcH7, and E6-AP has been analyzed.
- Expression profile of E6-AP has been compared with that of ER expression.
- Expression profile of UbcH7 has been compared with that of ER expression.
- Generation of the expression plasmids for overexpression of UbcH7 and E6-AP has been completed.
- The biological activities of His-tagged UbcH7 and E6-AP has been analyzed.
- Induciable expression vectors for UbcH7 and E6-AP have been constructed.

Reportable Outcomes

The ongoing work described here was presented as a poster and an abstract at the Annual Endocrine Society Meeting (June 2001), in Denver Colorado (see appendix 2) and at the Annual Endocrine Meeting (June 2002), in San Francisco (see appendix 3). An article regarding the roles of coactivators, including E6-AP, in breast cancer, has been published in Breast Cancer Research in June 2002 (see appendix 4).

Conclusions

We have successfully analyzed the expression of UbcH7, E6-AP and ER in different breast cancer cell lines. Additionally, we have also examined the effects of steroids on the expression profile of UbcH7, E6-AP and ER. In order to study the expression profile of UbcH7, E6-AP and ER in human breast tumors, we have examined 56 advanced stage human breast cancer biopsy samples by Western blot; 51 samples by Immunhistochemistry, and 27 samples by Immunofluorescence. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. Furthermore, E6-AP is down regulated in breast tumors, specifically low in stage IIB. However, we did not observe any correlation between the expression of UbcH7 and ER. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis.

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Appendices

1. Figures 1-20

2. Abstract 1

3. Abstract 2

4. Article

Figure 1: Expression analysis of UbcH7 in different cell lines (Hela, MCF7, T47DZR-75-1 and MDA-MB-231). Cells were grown on a chamber slide for 24 hrs and UbcH7 expression was analyzed by fluorescent immunocytochemistry using an anti-UbcH7 antibody. Positive signal for UbcH7 is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. UbcH7, UbcH7 expression profile; DAP1, DAP1 staining for nucleus.

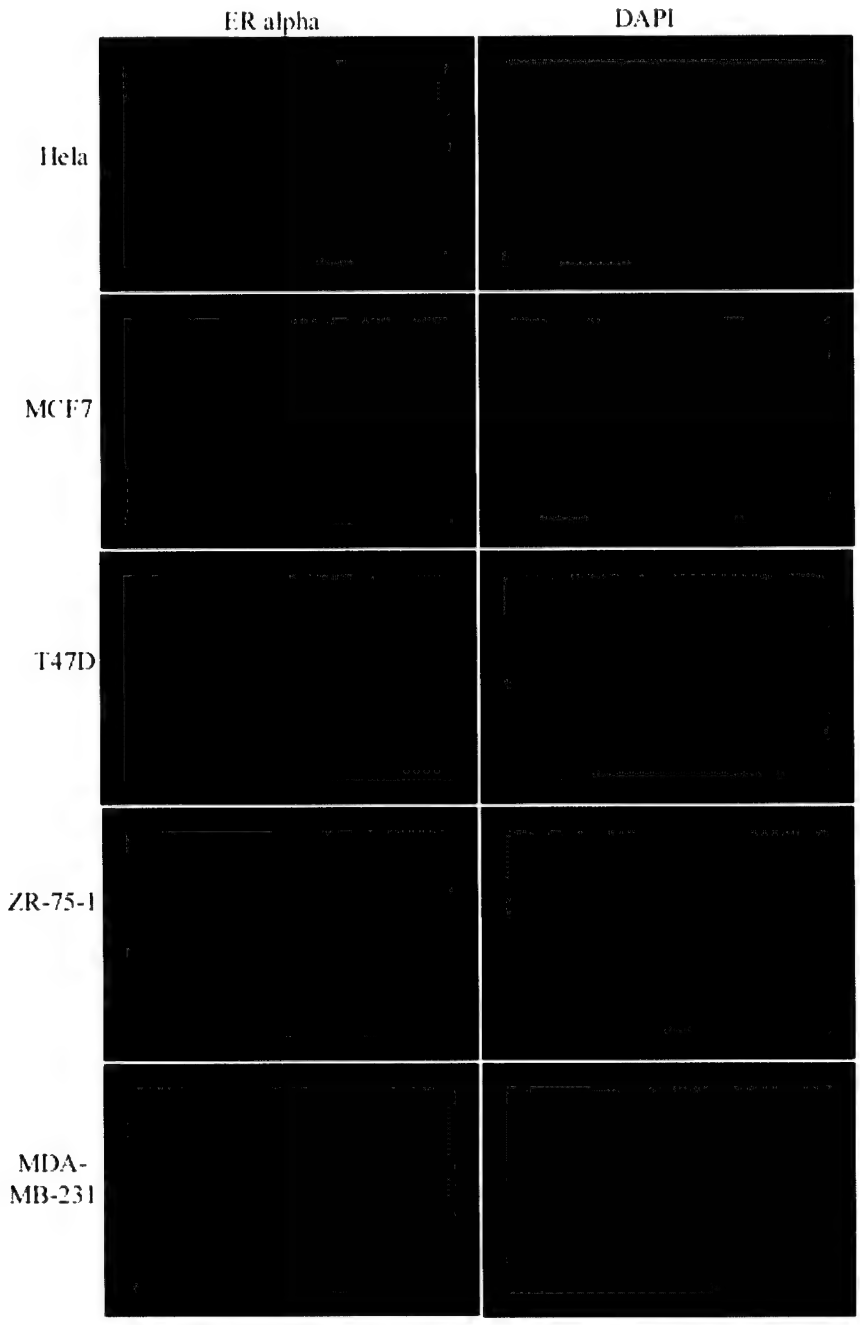

Figure 2: Expression analysis of ER-alpha in different cell lines (Hela, MCF7, T47D, ZR-75-1and MDA-MB-231. Cells were grown on a chamber slide for 24 hrs and ER-alpha expression was analyzed by fluorescent immunocytochemistryusing an anti-ER-alpha antibody. Positive signal for ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. ER-alpha, ER-alpha expression profile; DAP1, DAP1 staining for nucleus.

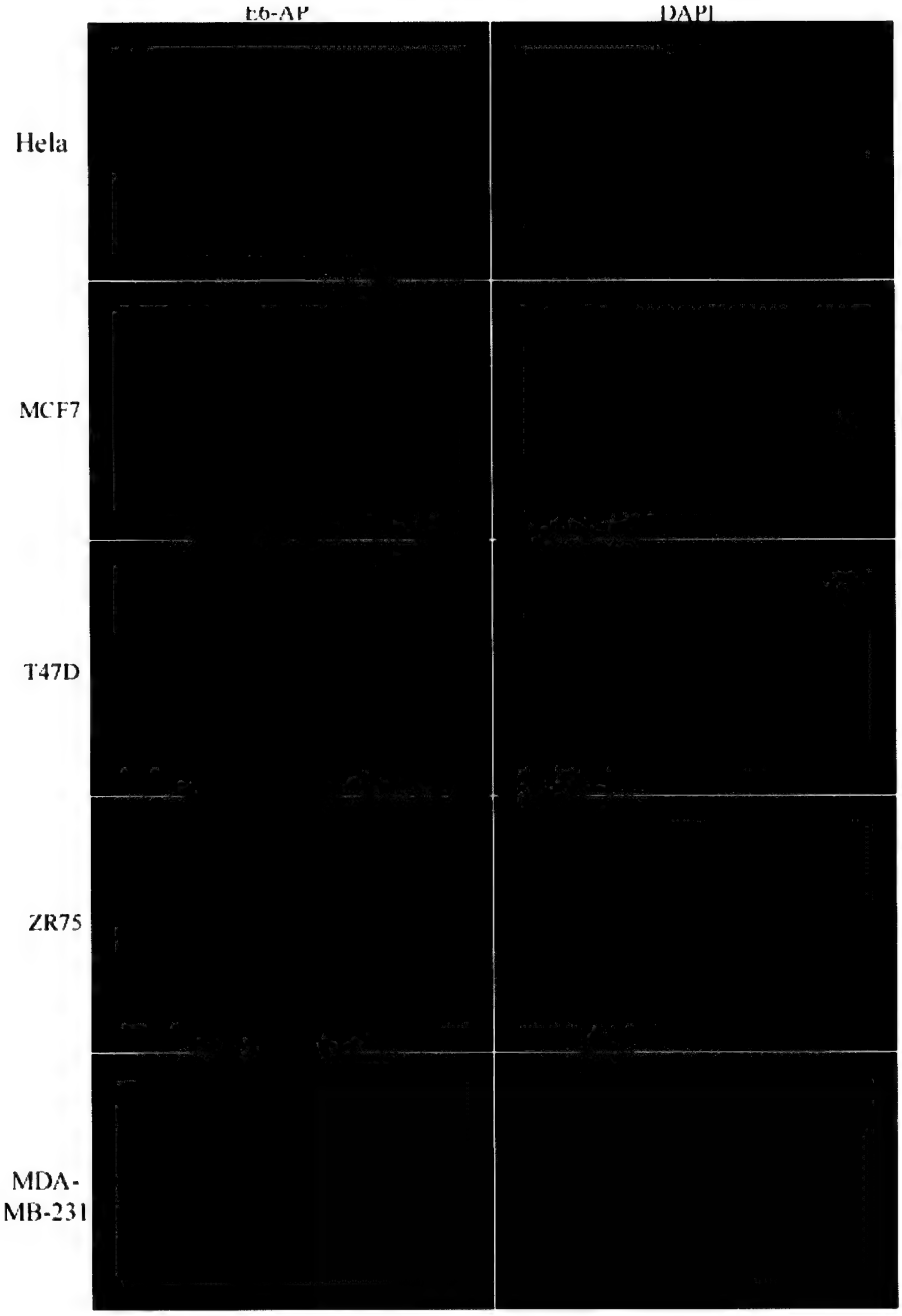

Figure 3: Expression analysis of E6-AP in different cell lines (Hela, MCF7, T47D, ZR-75-1 and MDA-MB-231. Cells were grown on a chamber slide for 24 hrs and E6-AP expression was analyzed by fluorescent immunocytochemistryusing an anti-E6-AP antibody. Positive signal for E6-AP is seen as (red) spots and nucleus is seen as (blue) spots in DAP1 staining. E6-AP, E6-AP expression profile; DAP1, DAP1 staining for nucleus.

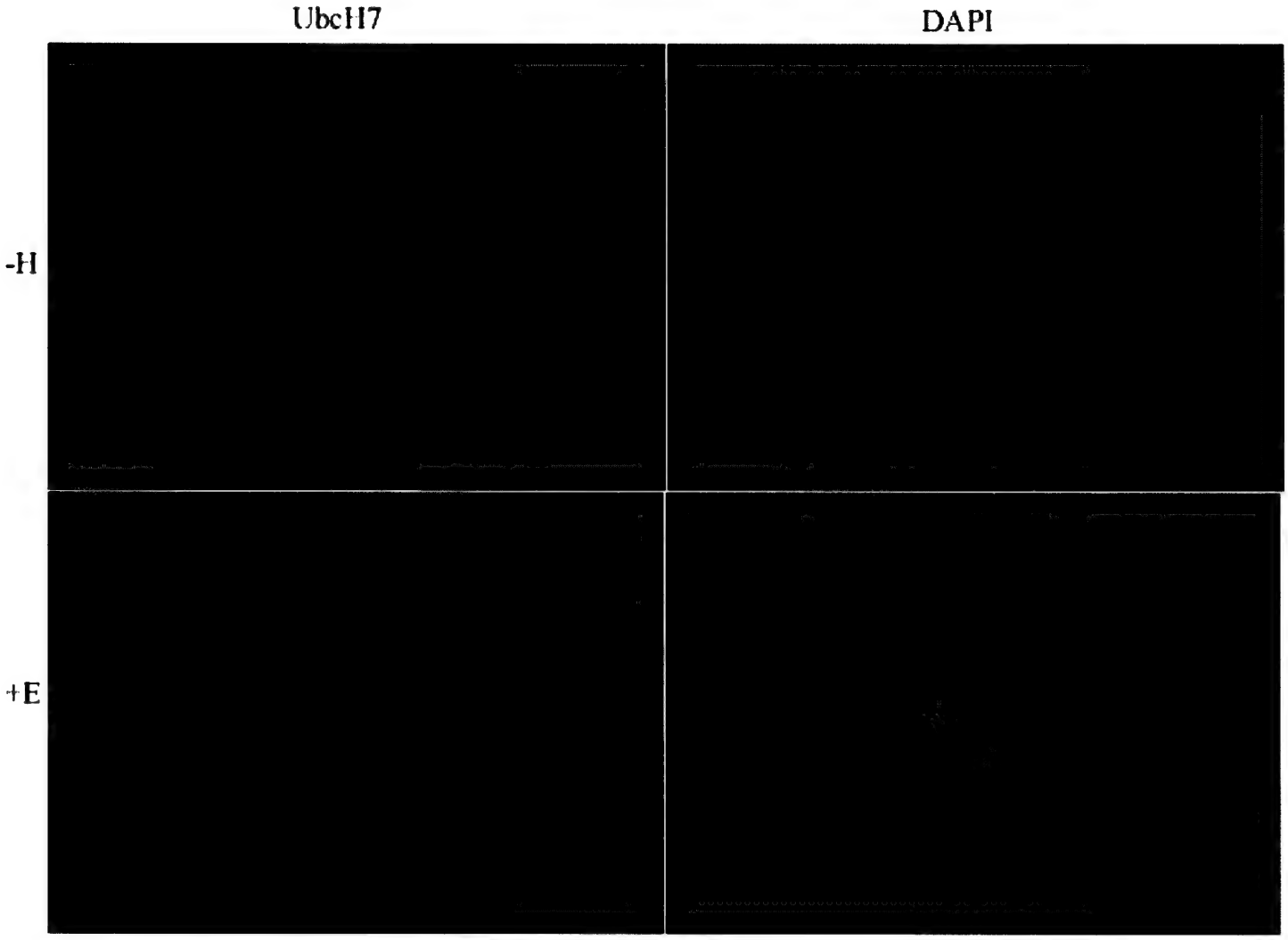

Figure 4: Effect of estrogen on the expression of UbcH7 in MCF7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous UbcH7 was analyzed by fluorescent immunocytochemistry using an anti-UbcH7 antibody. Positive signal for UbcH7 is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. UbcH7, UbcH7 expression profile; DAP1, DAP1 staining for nucleus.

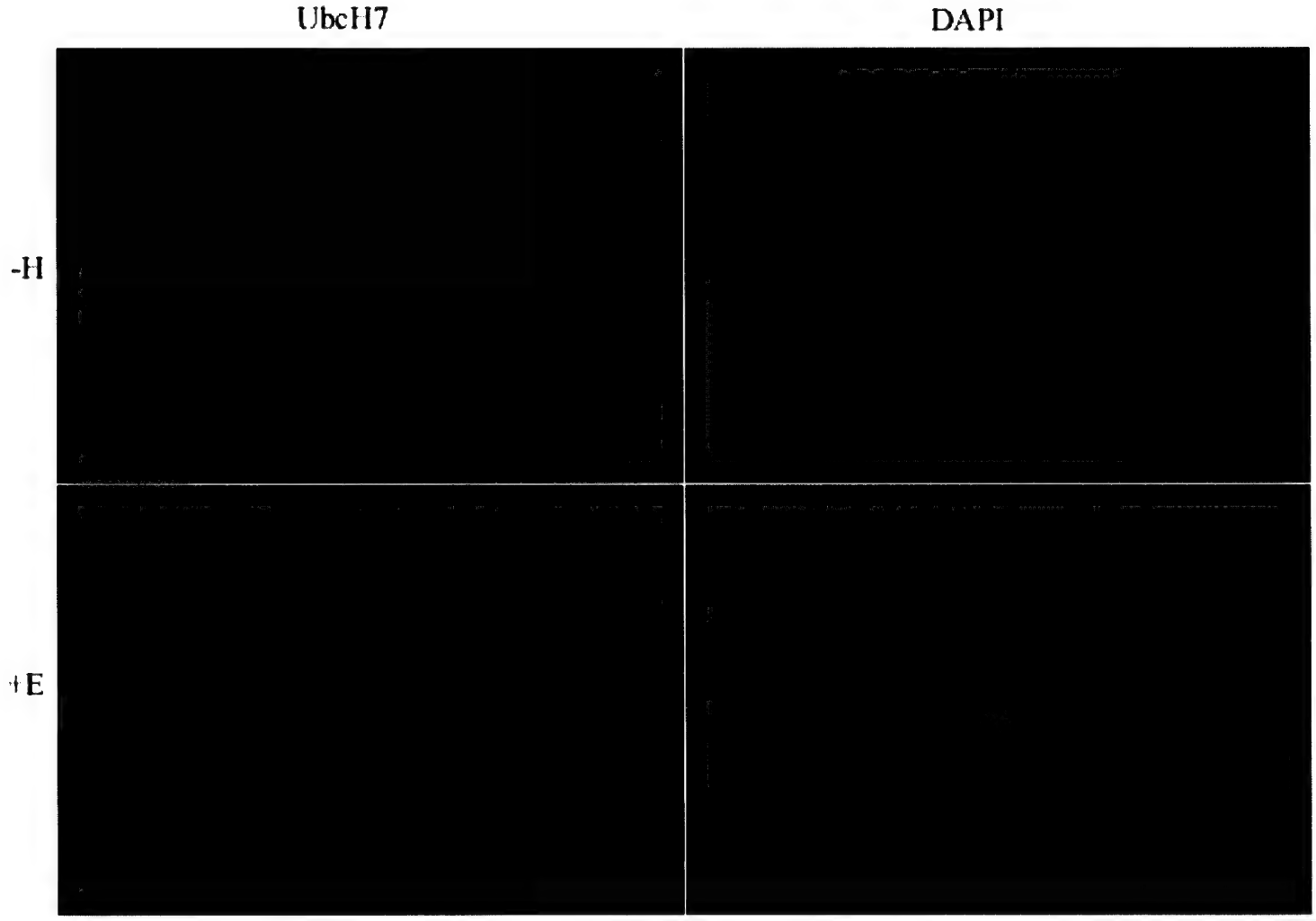

Figure 5: Effect of estrogen on the expression of UbcH7 in T47D cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous UbcH7 was analyzed by fluorescent immunocytochemistry using an anti-UbcH7 antibody. Positive signal for UbcH7 is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. UbcH7, UbcH7 expression profile; DAP1, DAP1 staining for nucleus.

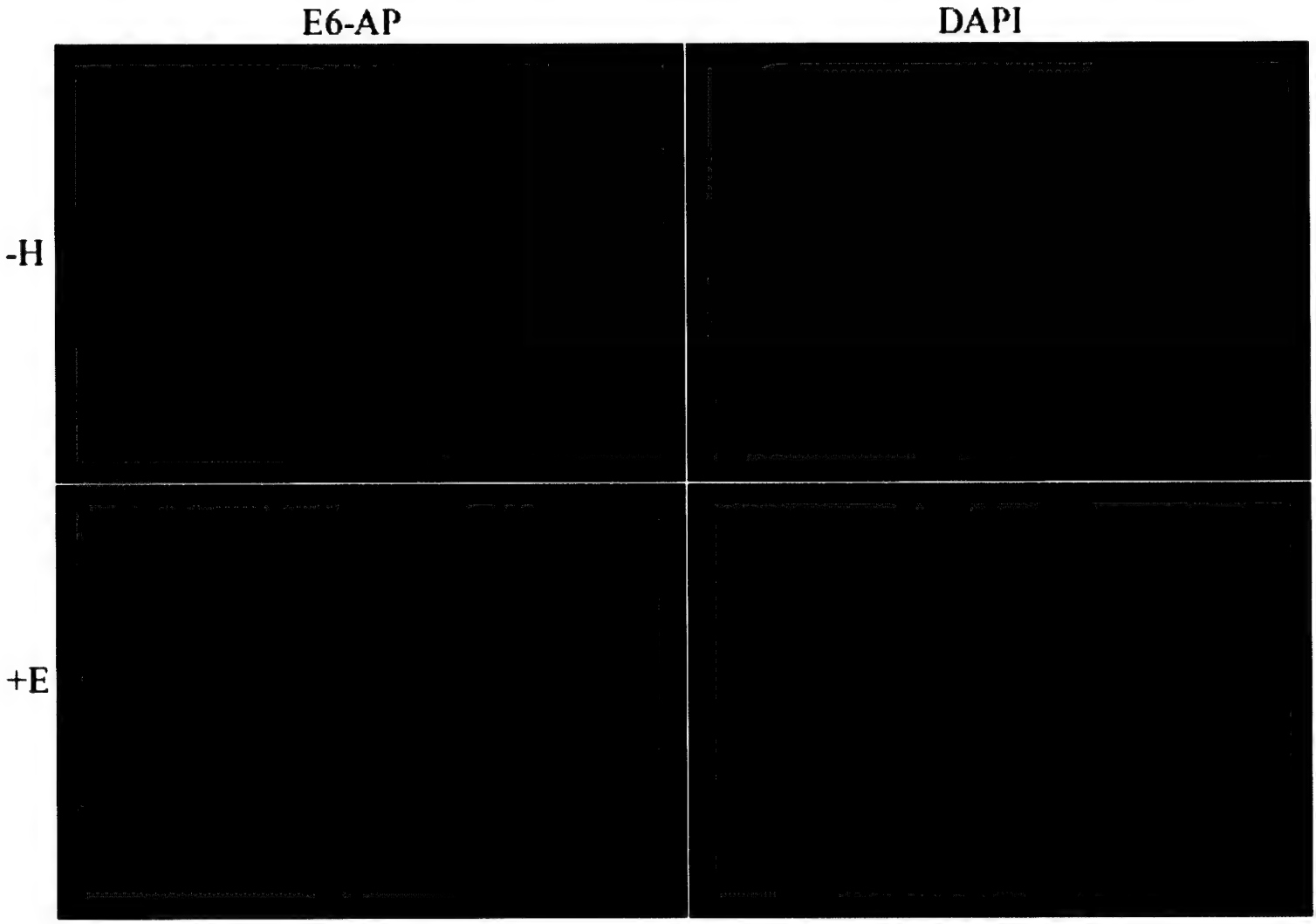

Figure 6: Effect of estrogen on the expression of E6-AP in MCF7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous E6-AP was analyzed by fluorescent immunocytochemistry using an anti-E6-AP antibody. Positive signal for E6-AP is seen as (red) spots and nucleus is seen as (blue) spots in DAP1 staining. E6-AP, E6-AP expression profile; DAP1, DAP1 staining for nucleus.

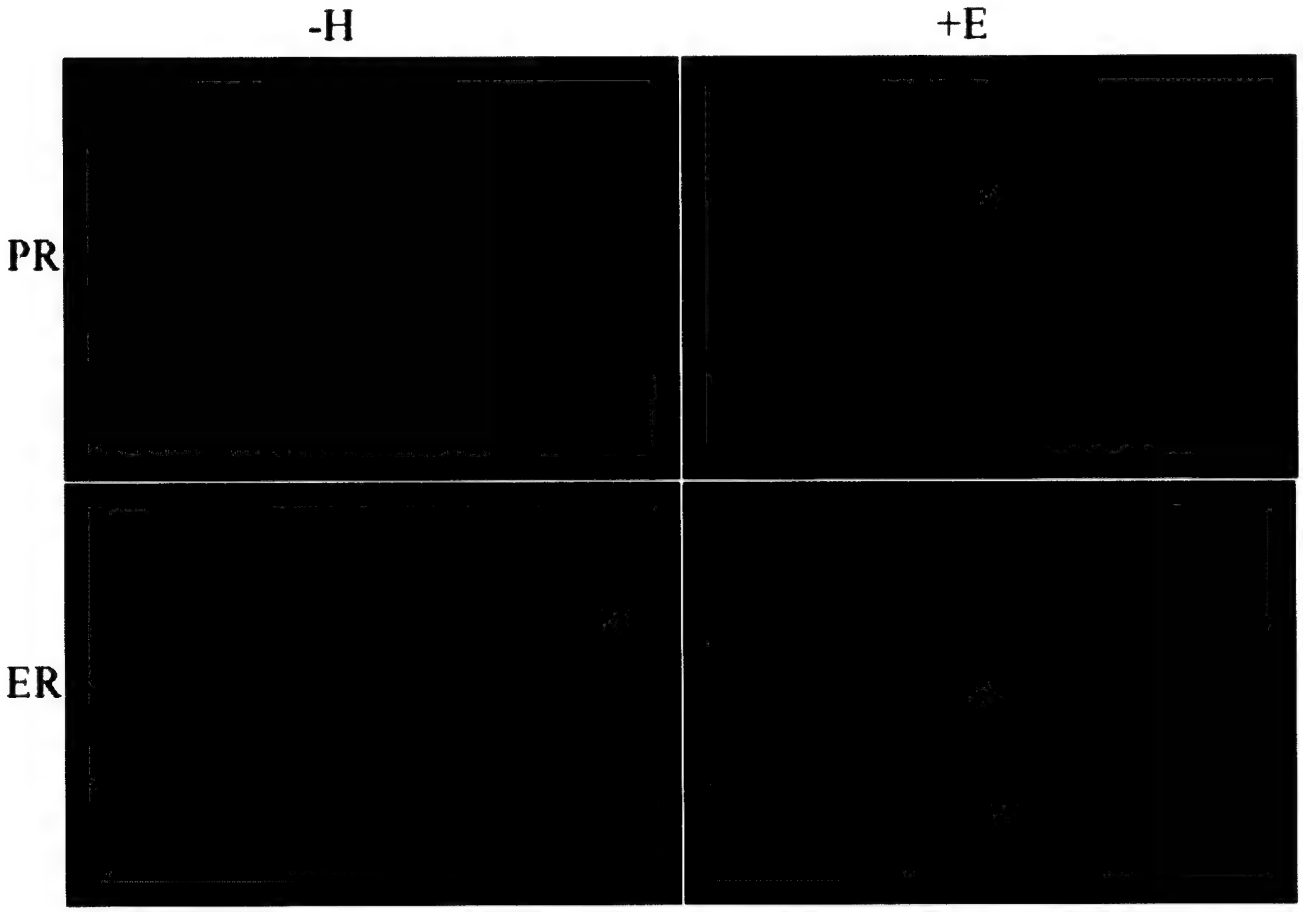

Figure 7: Effect of estrogen on the expression of PR and ER-alpha in MCF7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous PR and ER-alpha was analyzed by fluorescent immunocytochemistry using anti-PR and anti-ER-alpha antibodies. Positive signal for PR and ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. PR, PR expression profile; ER, ER-alpha expression profile; DAP1, DAP1 staining for nucleus.

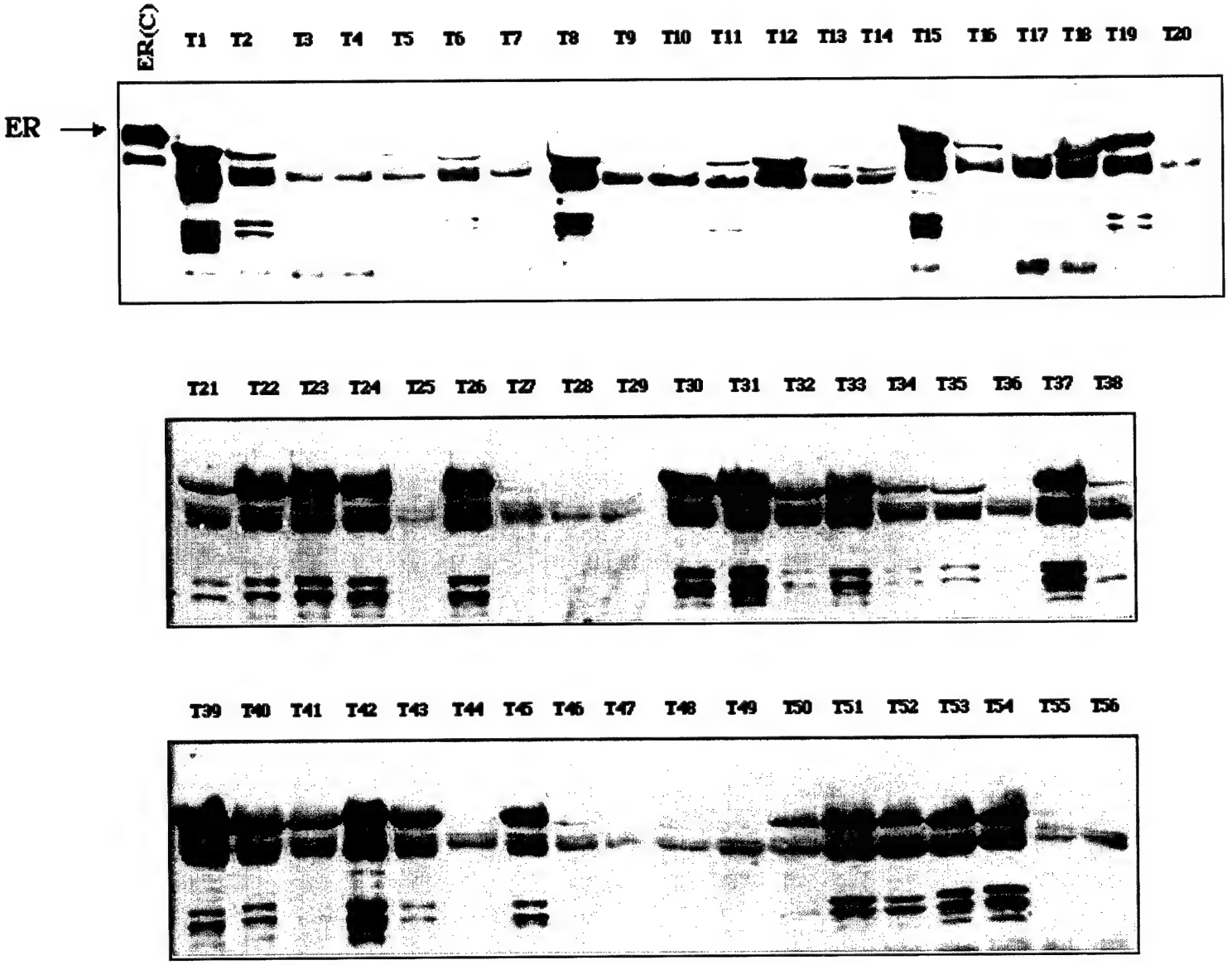

Figure 8: Expression analysis of ER-alpha in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of ER-alpha was analyzed by Western blot analysis using an anti-ER antibody. C, Purified ER protein was used as a control. T1-T56 represent different tumor samples.

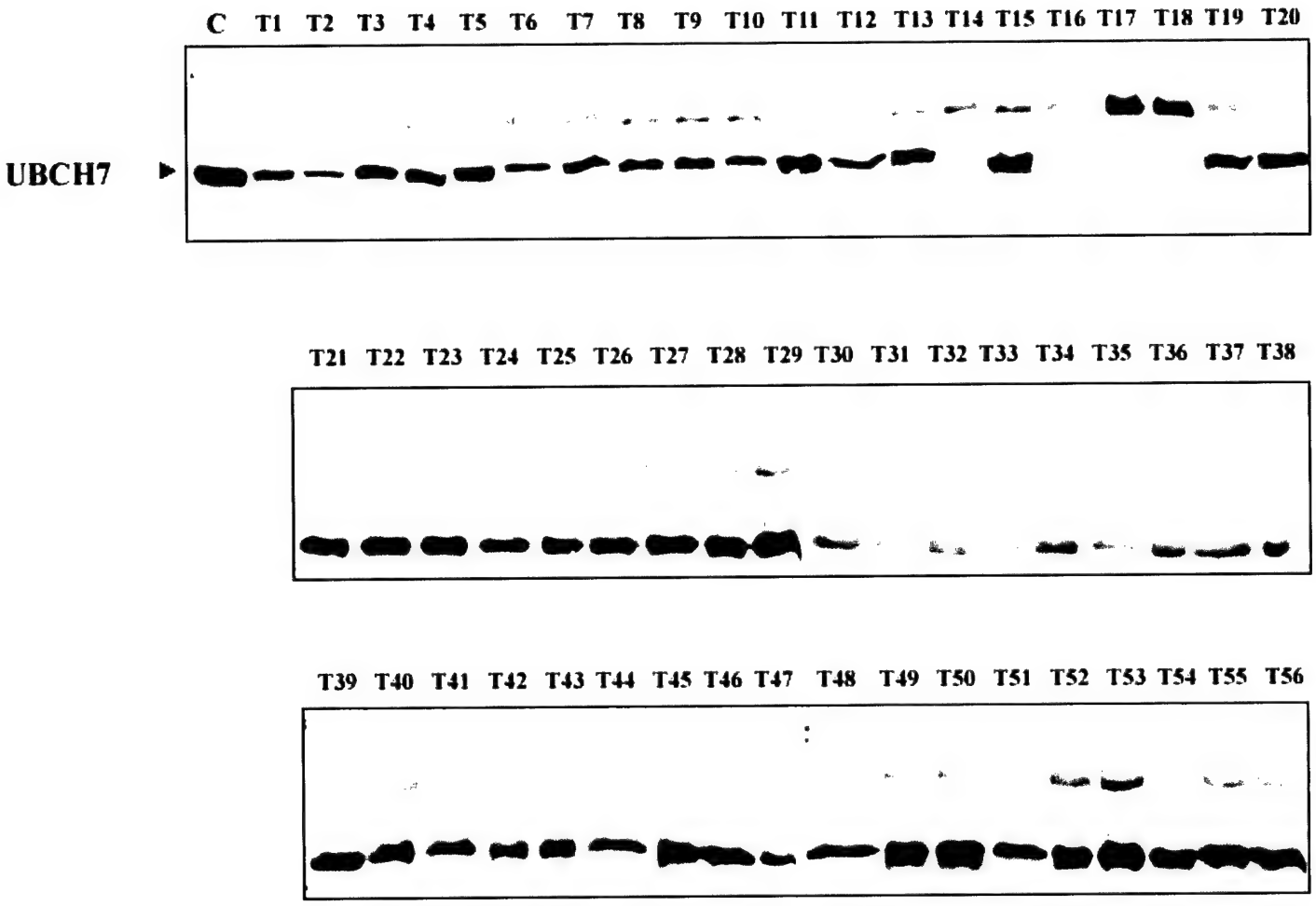

Figure 9: Expression analysis of UbcH7 in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of UbcH7 was analyzed by Western blot analysis using an anti-UbcH7 antibody. C, Purified UbcH7 protein was used as a control. T1-T56 represent different tumor samples.

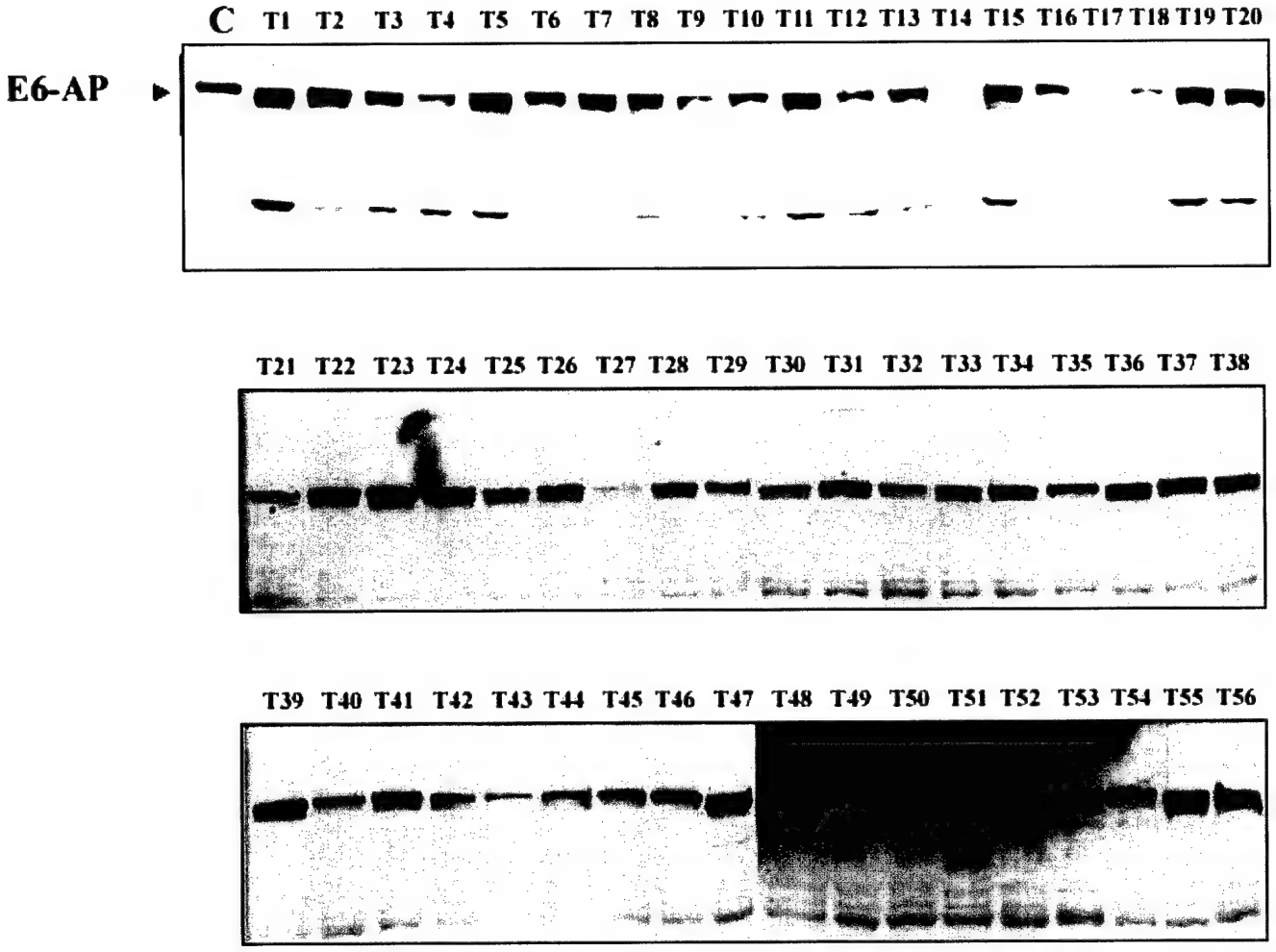

Figure 10: Expression analysis of E6-AP in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of E6-AP was analyzed by Western blot analysis using an anti-E6-APantibody. C, Purified E6-AP protein was used as a control. T1-T56 represent different tumor samples.

Figure 11: Expression analysis of p53 in human breast tumors. Tissue extracts were prepared from biopsy tumor samples and the expression of p53 was analyzed by Western blot analysis using an anti-p53 antibody. C, Purified p53 protein was used as a control. T1-T20 represent different tumor samples.

Tumor #	E6-AP	ER	UbcH7	Tumor #	E6-AP	ER	UbcH7	Tumor #	E6-AP	ER	UbcH7
1	++++	++++	++	21	+	+	+++	41	++	+	+
2	+++	+	+	22	+++	++	+++	42	++	++++	+
3	++	-	+++	23	++++	++++	+++	43	+	++	+
4	+	-	+++	24	+++	+++	++	44	+	++	+++
5	++++	-/+	++++	25	++	-	+++	45	++	++	+++
6	++	-/+	+	26	++	++++	++	46	+++	-/+	+++
7	++	-	++	27	-/+	-/+	+++	47	+++	-/+	++
8	+++	+++	++	28	++	-/+	++++	48	+	-/+	++
9	+	-/+	++	29	++	-/+	++++	49	++	-/+	+++
10	++	-/+	++	30	++	+++	-/+	50	++++	+	+++
11	+++	+	++++	31	++++	++++	-/+	51	++++	+++	++
12	++	++	+++	32	++	+	-/+	52	++++	+	++++
13	++	-/+	+++	33	+++	++	-/+	53	+++	++	++++
14	-/+	-/+	-/+	34	+++	+	-/+	54	+++	++	+++
15	++++	++++	+++	35	++	+	-/+	55	++++	-/+	+++
16	++	-/+	-/+	36	+++	-	-/+	56	+++	-/+	+++
17	-/+	-	-/+	37	+++	++++	-/+				
18	+	+	-/+	38	+++	+	-/+				
19	++++	++	++	39	++++	+++	++				
20	++++	-	++++	40	+	++	+				

Figure 12: Correlation of the expression of E6-AP and UbcH7 with that of ER-alpha in breast tumors. Expression levels of E6-AP, ER-alpha and UbcH7 from Western blot analysis was artificially graded according to the density of the bands. "-" represents negative expression, whereas "-/+" represents very low expression. From "+" to "++++" represent the gradually increasing levels of expression from low to high. Sprearman Rank Correlation Coefficient for the expression of E6-AP with that of ER-alpha is 0.38, p=0.004. However, there is no correlation between UbcH7 and ER-alpha expression and UbcH7 and E6-AP expression.

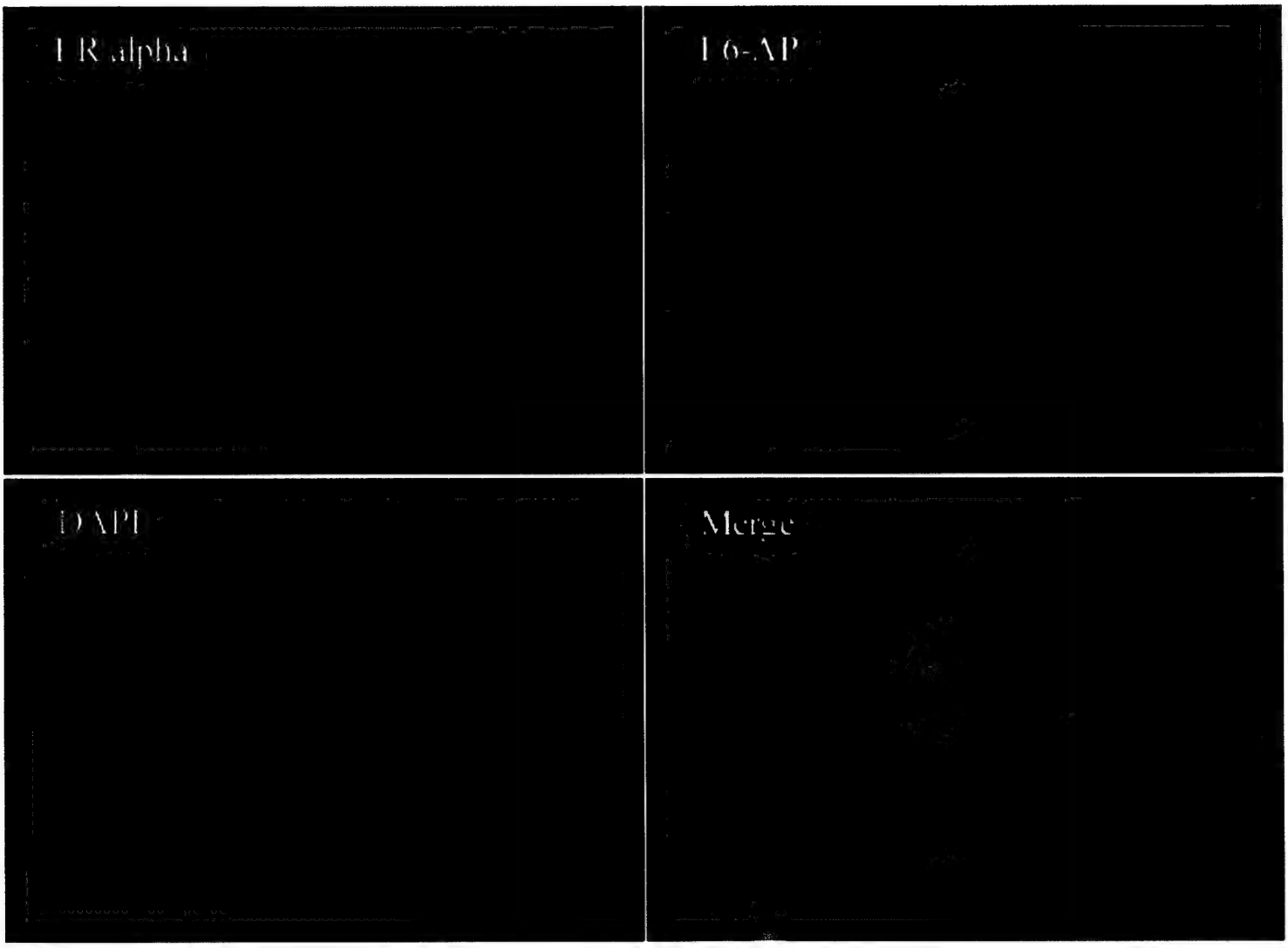

Figure 13: Colocalization of E6-AP and ER-alpha in T47D human breast cancer cell line by immunocytochemistry. Cells were grown on a chamber slide for 24 hours and the expression of endogenous E6-AP and ER-alpha was analyzed by fluorescent immunocytochemistry using either an anti-E6-AP or ER-alpha antibody. Positive signal for E6-AP is seen as (red) spots, ER-alpha is seen as (green) spots and nucleus is seen as (blue) spots in DAP1 staining. Yellow spots (merge) indicate colocalization of ER-alpha and E6-AP. ER-alpha, ER-alpha expression profile; E6-AP, E6-AP expression profile; DAP1, DAP1 staining for nucleus.

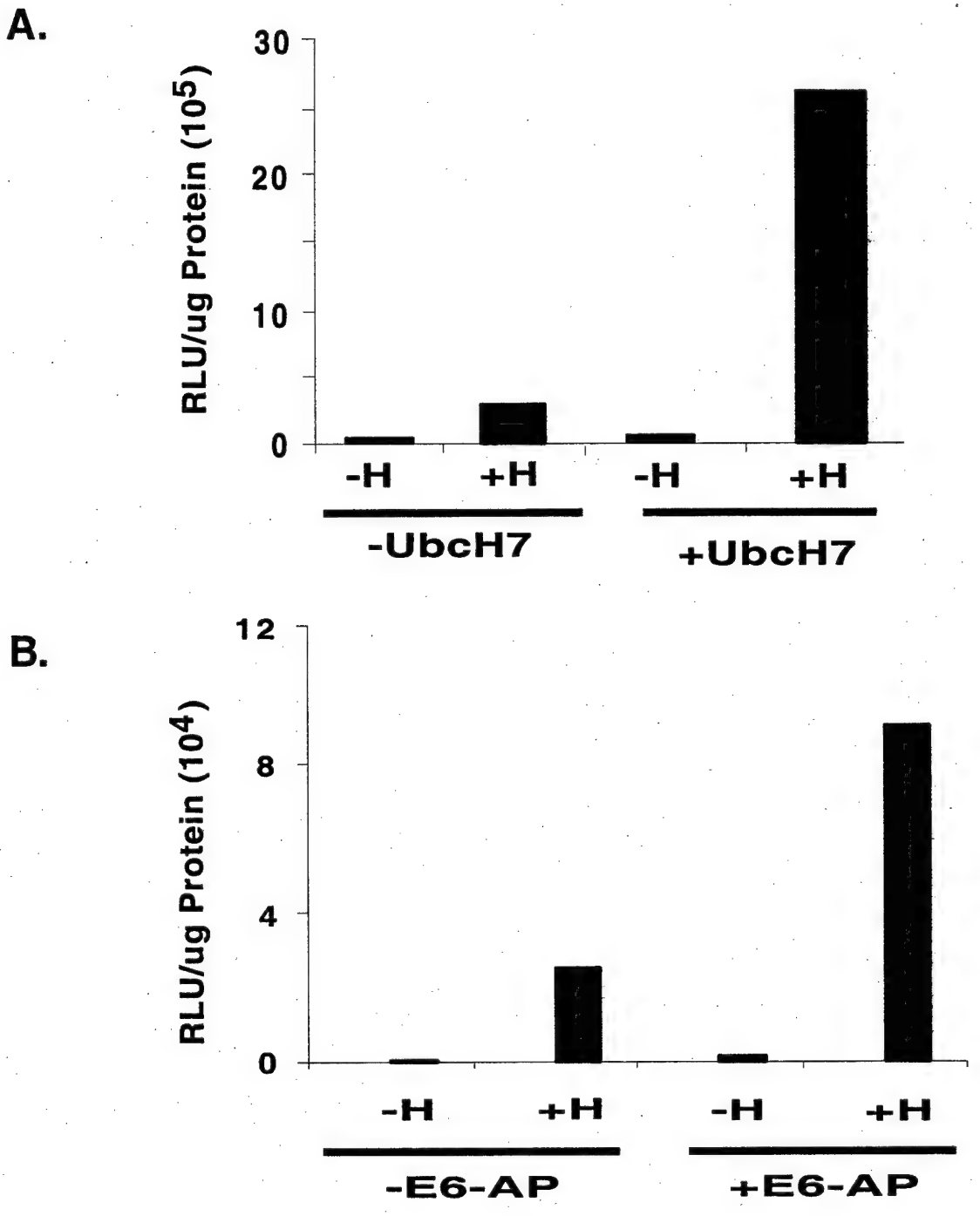

Figure 14: His-tagged UbcH7 and E6-AP were able to coactivate PR activity. Hela cells were transiently transfected with progesterone receptor expression plasmid and progesterone-responsive reporter plasmid in the absence or presence of his-tagged UbcH7 or E6-AP expression plasmid. The cells were treated with either vehicle (-H) or 10^{-7}M Progesterone (+H). The data is presented as relative light units/ug protein (RLU/ug Protein).

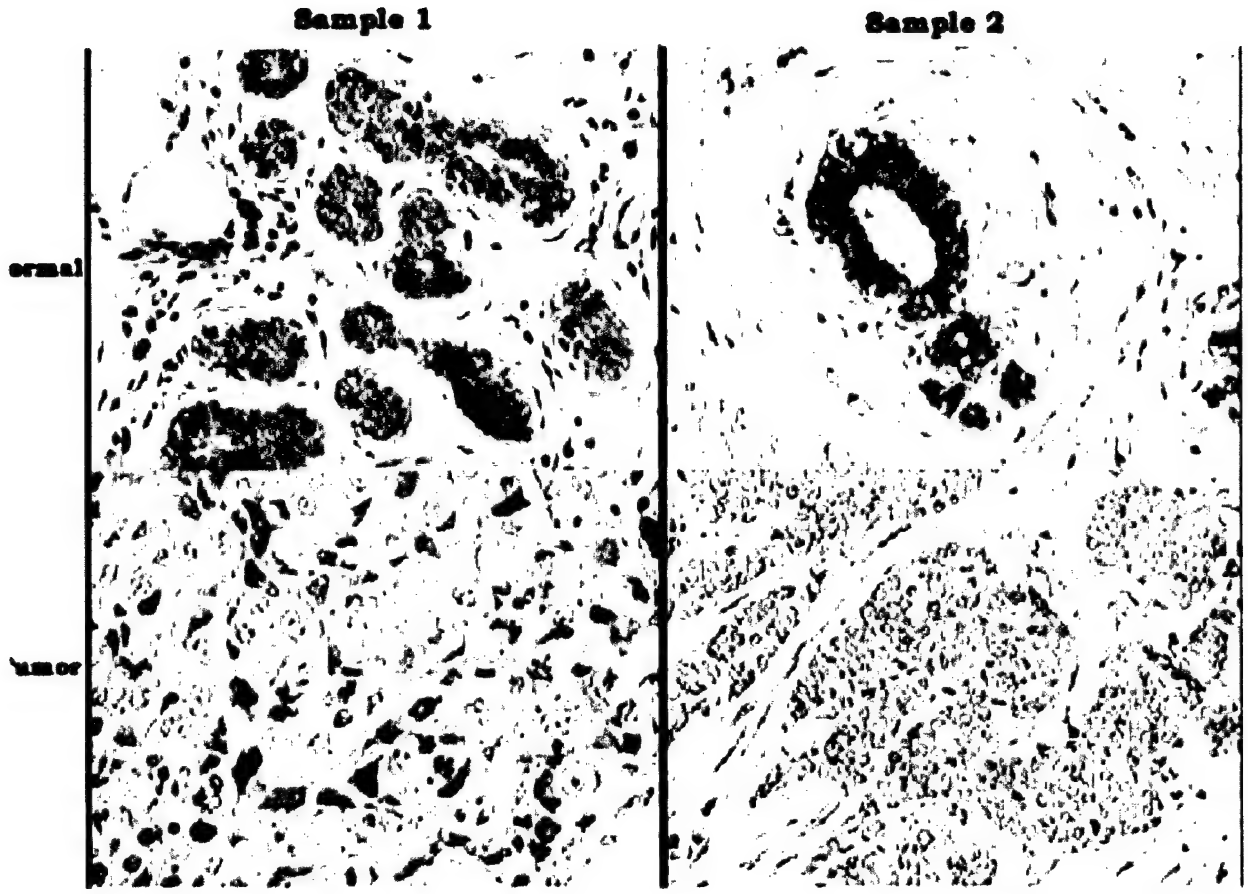

Figure 15. Immunohistochemical analysis of the expression of E6-AP in normal and malignant human breast tissues. The expression pattern of E6-AP in 12 human breast tumors (grade 1 to 3) and their adjacent normal tissues were analysed by means of immunohistochemistry using an anti-E6-AP antibody. Positive signal for E6-AP is seen as brown spots; nucleus is seen as blue color as the result of counterstaining by hematoxylin. This figure shows the result of two paired samples.

Relative Expression of E6-AP in Human Mammary Tumors and Their Adjacent Normal Tissues

		NORMAL TISSUES		Total
		High	Low	
TUMOR	High	2	0	2
	Low	8	2	10
	Total	10	2	12

*Chi-Square Test, $p<0.025$

Figure 16. The distribution of different levels of E6-AP expression in normal and malignant human breast tissues. Comparing the expression level of E6-AP in each pair of samples,there are four kinds of outcomes: equally high in both tumor and normal tissues; equally low in both tumor and normal tissues; high in tumor and low in normal tissues; low in tumor and high in normal tissues. It is noticed that the majority of the samples (8 out of 12) express lower levels of E6-AP in tumors in comparison to their adjacent normal tissues. Chi-Square test shows that the differences between the four groups are statistically significant ($p<0.025$).

Stage I	Stage IIA	Stage IIB	Stage IIIA	Stage IIIB
1	0.5	0.5	1.5	
1.5	1	1	2	
1.5	1	1	2	
2	1	1	2	
2	1.5	1	2	
2	1.5	1.5	2.5	
2	2	2		
2.5	2			
3	2			
	2			
	2			
	2.5			
	3			
	3			
N1=9	N2=14	N3=7	N4=6	
$\bar{X}$1=1.94	X2=1.78	X3=1.14	X4=2.00	

Figure 17. The expression of E6-AP in different stages of human breast tumors. A tumor tissue array with 36 human breast cancer samples in one slide,(including tumors from stage I to stage III) were used to analyse the levels of E6-AP expression by immunoohistochemistry using an anti-E6-AP antibody. Since E6-AP is universally expressed in the epithelial cells, the levels of expression from immunohistochemical analysis were artificially graded according to the intensity of the brown color in each sample. "0" represents negative expression, whereas "0.5" represents very low expression. From "1" to "4" represent the gradually increasing levels of expression from low to high. Normal tissue was included in the experiment as a positive control, which is always express high level of E6-AP. E6-AP is differently expressed in different stages of breast tumors. N, numbers of samples in each stage; X, average expression level of E6-AP in the stage.

Comparison of the Expression Level of E6-AP between Different Stages of Breast Tumors by Wilcoxon Rank-sum Test

Stages Compared	Rank-sum T	n1,n2-n1	*P* value
I & IIB	32	7, 2	<0.01*
IIA & IIB	52.5	7, 7	>0.05
IIIA & IIB	59.5	6, 1	<0.01*
I & IIA	115	9, 5	>0.1
I & IIIA	50.5	6, 3	>0.1

Figure 18. Statistical analysis of the expression levels between different stages of breast tumors. Based on the distribution of E6-AP in different stages of breast tumors (shown in Figure 17), the differences of the levels of E6-AP expression between different stages of breast tumors were analyzed by Wilcoxon rank-sum test. Since Figure 17 suggested that the expression of E6-AP decreased gradually from stage I to stage IIB, and then it goes up again in stage IIIA, we compared the differences of expression between stage IIB with that of every other stage. The expression of E6-AP in stage I, stage IIIA were significantly different from that of stage IIB, suggesting that stage IIB is the lowest point in E6-AP expression.

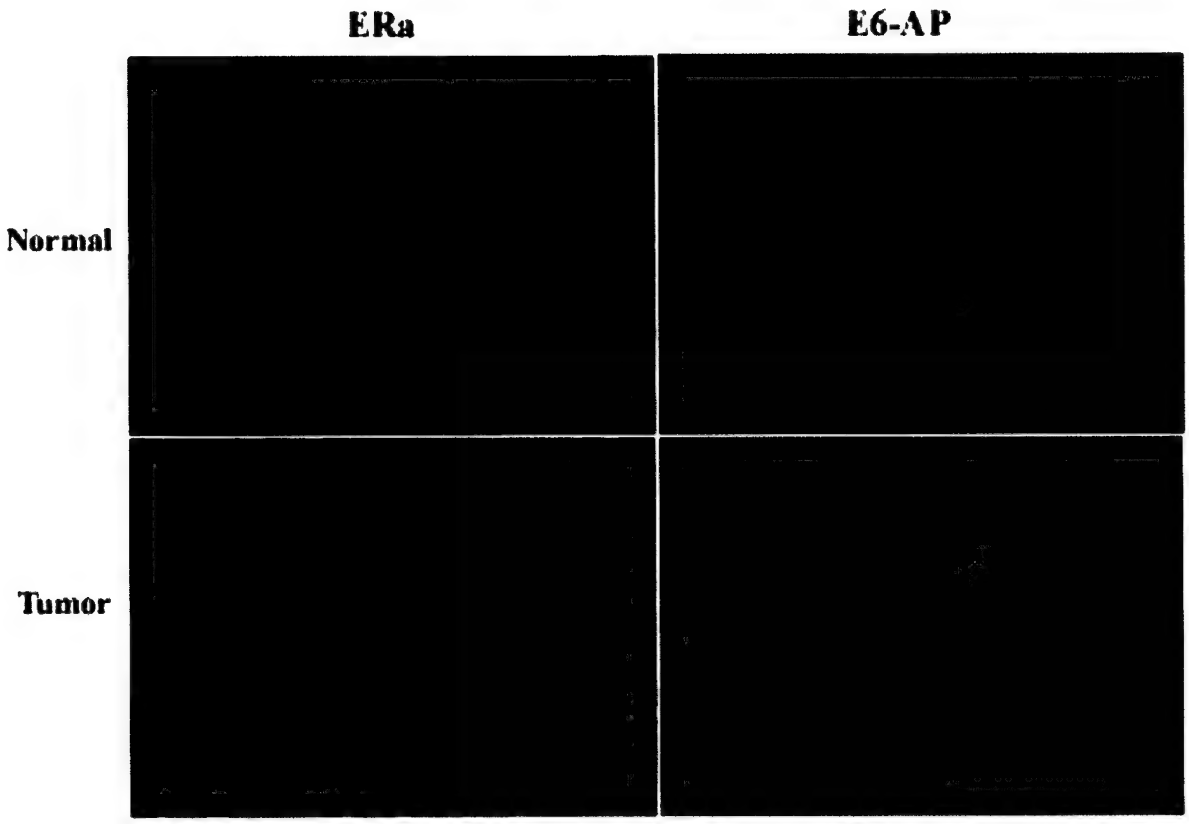

Figure 19. The expression of E6-AP is inversely correlated with that of ERa. The expression patterns of E6-AP and ERa in breast tumors and normal tissues were studied by means of dual immunofluorescent staining using antibodies against E6-AP and ER alpha. This picture is a typical example from the 8 pairs of samples studied. E6-AP is seen as red spot, while ERa is seen as green spot. In the normal breast tissues, ERa is expressed in the nuclei of epithelial cells in a discontinuous manner, whereas E6-AP is highly and broadly expressed in the epithelial cells, mostly in the cytoplasm. In comparison with its normal controls, the expression level of E6-AP is lower in tumors, while the expression of ERa is higher. Altogether, 5 out of 8 tumors that have lower levels of E6-AP express higher levels of ERa.

#	E6-AP	ERα	#	E6-AP	ERα
1	2	4	11	0	3
2	3	3	12	2	1
3	0.5	3	13	0	3
4	1	2	14	0	0
5	1.5	3	15	2	2
6	1	3	16	2	3.5
7	0	0	17	2	4
8	0.5	3	18	0.5	1
9	1	3	19	0.5	0
10	1	3			

*Spearman Rank Correlation Coefficient r=0.503, $p<0.05$

Figure 20. Correlation of the expression of E6-AP with that of ER-α in breast tumors. Expression levels of E6-AP and ERα from immunohistochemical analysis were artificially graded according to the intensity of the respective colors; red for E6-AP and green for ERα. ERα is expressed in the nucleus, whereas E6-AP is expressed mostly in the cytoplasm. "0" represents negative expression and "0.5" represents very low expression. From "1" to "4" represent the gradually increasing levels of expression from low to high. Spearman Rank Correlation Coefficient for the expression of E6-AP with that of ERα is 0.503, $p<0.05$.

Abstract View

P1-241

INVOLVEMENT OF STEROID HORMONE RECEPTOR COACTIVATORS, E6-AP AND UBCS, IN THE DEVELOPMENT OF BREAST TUMORS

X Gao[1], GM Clark[2], F Yan[1], Z Nawaz[1]

[1] Molecular and Cellular Biology, Baylor College of Medicine, Houston., Texas , [2] Breast Center, Baylor College of Medicine, Houston, Texas

Steroid hormones, estrogen and progesterone, are known to play a major role in the development of breast tumors by functioning through their cognate intracellular receptors, estrogen receptor (ER), and progesterone receptor (PR), respectively. Coregulators of steroid hormone receptors are important mediators of steroid receptors' function. Changes in the expression of these coactivators may contribute to mammary gland tumorigenesis. Recently, our laboratory identified several ubiquitin pathway enzymes, such as E6-associated protein (E6-AP) and ubiquitin conjugating enzymes (UBCs), as coactivators of steroid hormone receptors. Seperately, it was reported that E6-AP was overexpressed in a spontaneous mouse model of mammary gland tumorigenesis. To study the expression profiles of E6-AP and UBCs in human breast tumors, we examined 56 advanced stage human breast cancer biopsy samples. We found a correlation between the expression of E6-AP and the expression of ER-alpha in these breast tumors using Western blot analysis. The Spearman Rank Correlation Coefficient was 0.38 and the p value was 0.004, indicating that this correlation was statistically significant. Furthermore, the expression of E6-AP also correlated with that of UbcH7 (p=0.002), although the latter did not correlate with the expression of ER-alpha (p=-0.16). Our data provide the first evidence of a relationship between steroid hormone receptors and their coactivators, E6-AP and UBCs, suggesting a possible role of these coactivators in mammary gland tumorigenesis. It also indicates that E6-AP may be a potential target for breast cancer therapy.

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Contact Author Xiuhua Gao, M.D., Ph.D.
Department/Institution: Molecular and Cellular Biology, Baylor College of Medicne
Address: One Baylor Plaza
City/State/Zip/Country: Houston, Texas, 77030, United States
Phone: 713-798-5269 **Fax:** 713-790-1275 **E-mail:** xgao@bcm.tmc.edu

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Title: E6-ASSOCIATED PROTEIN, E6-AP, IS INVOLED IN THE TUMORIGENESIS OF BOTH MAMMARY GLAND AND PROSTATE GLAND

Xiuhua Gao [1]*, Syed K. Mohsin [2], Thomas M. Weeler [3], Feng Yan [1] and Zafar Nawaz [1].
[1]Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, 77030; [2]Breast Cancer Center, Baylor College of Medicine, Houston, Texas, 77030 and [3]Scott Department of Urology, Baylor College of Medicine, Houston, Texas, 77030.

Nuclear receptor coactivators play a major role in modulation of estrogen (ER) and androgen (AR) receptor functions in breast and prostate, respectively. E6-associated protein (E6-AP) is an E3 ubiquitin-protein ligase involved in the selective protein degradation pathway, ubiquitin-proteasome pathway. It has also been characterized as a coactivator since it can potentiate the transcriptional activity of nuclear hormone receptors, such as ER, AR, progesterone receptor (PR) and glucocorticoid receptor (GR). Based on these facts, we hypothesized that expression of E6-AP may alter during breast/prostate tumorigenesis. To test this hypothesis, we conducted a pilot study to evaluate the expression profile of E6-AP by immunohistochemistry and immunofluorescence. 12 breast cancers (Grade 1 to 3) and 10 prostate cancers (Gleason scores 4 to 6) and their matched adjacent normal breast/prostate tissues were used for the analysis. While high level expression of E6-AP was consistently observed in normal epithelial cells of both mammary gland and prostate glands, decreased expression was found in 8 out of the 12 breast cancers (Chi-Square, $p<0.025$) and 8 out of the 10 prostate tumors (Chi-Square, $p<0.05$). Furthermore, of those tumors which had decreased level of E6-AP, 7/8 breast tumors expressed higher level of ER and 5/8 prostate tumors expressed higher level of AR, in comparison to their respective normal tissues. These data suggested that the alteration of E6-AP expression may have a role in the altered estrogen/androgen action occurring during tumorigenesis. Currently, we are expanding this study to a larger sample size to confirm these

results. We are also planning to establish stably transfected cancer cell lines which overexpress either wild-type or ubiquitin-protein ligase mutant E6-AP. By analyzing these cell lines' growth properties in vitro and their tumorigenicity in vivo, we may know whether the coactivator function or the E3-ubiquitin ligase function of E6-AP is involved in tumorigenesis.

Keyword 1: Steroid receptor coactivator (SRC)
Keyword 2: Ubiquitin
Keyword 3: Tumorigenesis

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Review

Progesterone receptors: animal models and cell signaling in breast cancer

Role of steroid receptor coactivators and corepressors of progesterone receptors in breast cancer

Xiuhua Gao and Zafar Nawaz

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, USA

Correspondence: Zafar Nawaz, Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. Tel: +1 713 798 6258; fax: +1 713 790 1275; e-mail: zn029815@bcm.tmc.edu

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Abstract

Progesterone, an ovarian steroid hormone, plays a key role in the development and function of the mammary gland, as it also does in the uterus and the ovary. The action of progesterone is mediated through its intracellular cognate receptor, the progesterone receptor (PR), which functions as a transcription factor that regulates gene expression. As with other nuclear receptors, coregulators (coactivators and corepressors) recruited by the liganded or unliganded PR, either to enhance or to suppress transcription activity, modulate the function of the PR. Mutation or aberrant expression of the coregulators might thus affect the normal function of the PR and hence disrupt the normal development of the mammary gland, which may lead to breast cancer.

Keywords: breast cancer, coactivator, corepressor, progesterone receptor

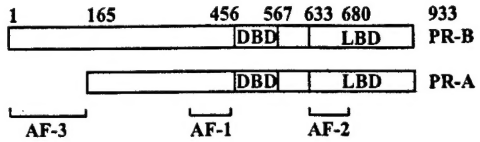
Introduction

The progesterone receptor (PR) is a member of the nuclear receptor superfamily, which specifically regulates the expression of target genes in response to the hormonal stimulus. In the absence of progesterone, the PR is sequestered in a nonproductive form associated with heat shock proteins and other cellular chaperones. In this state, the PR is considered unable to influence the rate of transcription of its cognate promoters [1]. On binding with progesterone, the PR undergoes a series of events, including conformational changes, dissociation from heat shock protein complexes, dimerization, phosphorylation, and nuclear translocation, which enables its binding to progesterone-response elements within the regulatory regions of target genes. The binding of PR to the progesterone-response elements is followed by the recruitment of coactivators and the basal transcription machinery, leading to the upregulation of target gene transcription.

The PR exists as two isoforms in most rodents and humans, PR-A and PR-B, which are produced from a single gene by translation initiation at two distinct start codons under the control of separate promoters [2]. The difference between PR-A and PR-B is that PR-A is a truncated form of PR-B. In humans, the N-terminal 164 amino acids of PR-B are missing in isoform PR-A. Detailed molecular dissection has identified two distinct activation function domains (AFs) within both PRs: AF-1, which is located in the N-terminal region, is ligand independent; AF-2, which is ligand dependent, is contained in the ligand-binding domain that is located in the C-terminal region. A DNA-binding domain and the hinge region are

AF = activation function domain; CBP = CREB-binding protein; E6-AP = E6-associated protein; ER = estrogen receptor; N-CoR = nuclear receptor corepressor; PR = progesterone receptor; RPF1 = receptor potentiation factor-1; RTA = repressor of tamoxifen transcriptional activity; SMRT = silencing mediator of retinoid and thyroid receptor; SRA = steroid receptor RNA activator; SRC = steroid receptor coactivator; Uba3 = ubiquitin-activating enzyme.

Figure 1



Schematic representation of the progesterone receptor PR-A and PR-B proteins. The DNA-binding domain (DBD), the ligand-binding domain (LBD) and activation function domains (AFs) are indicated.

mapped to the central region of both receptors. Furthermore, a unique activation function domain, AF-3, is contained in the upstream segment of PR-B that is missing in PR-A (Fig. 1).

Progesterone and estrogen are essential regulators of female reproductive activity. Through their cognate receptors, estrogen and progesterone regulate the normal development of the ovary, the uterus and the mammary gland, and play key roles in the tumorigenesis of these tissues. It has been demonstrated by estrogen receptor (ER) and PR knockout mice that estrogen controls the early ductal morphogenesis of the mammary gland, whereas progesterone controls ductal branching and alveolar development of the mammary gland during pregnancy [3].

Although the two forms of the PR have similar structures and are identical in DNA and ligand binding, *in vitro* studies using a reconstituted progesterone-responsive transcription system in mammalian cells revealed that PR-A and PR-B are not functionally identical. In most cases, PR-B acts as a potent activator of transcription of target genes, whereas PR-A acts as a dominant repressor of transcription of PR-B as well as a few other nuclear receptors [4]. The AF-3 domain in PR-B is partially responsible for the higher transcriptional activity of PR-B relative to PR-A. Moreover, an inhibitory function domain that is located in the N-terminus of both receptor isoforms has been identified [2]. This inhibition function domain can inhibit the activity of AF-1 and AF-2 but not that of AF-3, which explains why PR-B is a potent activator of transcription. The inhibition function domain is functionally independent and is transferable; when placed upstream of the ER, the inhibition function domain can also suppress ER activity.

The relative expression of PR-A and PR-B in the target tissues is dependent on species, cellular context, and the physiological and hormonal status. The ratio of PR-A to PR-B in specific tissues or cell types defines the physiological and pharmacological responses to progesterone. In the mammary gland, the ratios of PR-A to PR-B are constant from puberty to pregnancy, although there are species differences [5]. From PR-A knockout mice, it has been demonstrated that PR-B is mainly responsible for the normal proliferative and differentiative responses of the mammary gland to progesterone, because PR-A knockout mice exhibit a similar phenotype to PR knockout mice [3]. Overexpression of PR-A over PR-B in transgenic mice results in extensive epithelial cell hyperplasia, in excessive ductal branching, and in a disorganized basement membrane. All these features are associated with neoplasia [6]. Furthermore, the development of the mammary gland in PR-B overexpressed transgenic mice is also abnormal [7], indicating that a regulated expression of PR-A and PR-B and the native ratios of the two isoforms are critical to the appropriate responsiveness of the mammary gland to progesterone. Consistent with the findings from animal studies, very low levels of PR-B and a consequently high PR-A:PR-B ratio were found in a significant proportion of human breast cancer samples [8]. Taken these findings together, it can be inferred that imbalance of PR-A versus PR-B may be associated with the development, progression or prognosis of breast cancer.

Coactivators are factors that can interact with nuclear receptors in a ligand-dependent manner and enhance their transcriptional activity. Corepressors are factors that interact with nuclear receptors and repress their transcriptional activity. Both types of coregulators are required for efficient modulation of target gene transcription by the PR [9]. Changes in the expression level and pattern of PR coactivators or corepressors, or mutation of their function domains, might therefore affect the transcriptional activity of the PR and hence cause disorders of its target tissues, including the mammary gland. The present review will describe the coactivators and corepressors that are involved in the transcriptional modulation of PRs, with emphasis on their roles in breast cancer development and progression.

Progesterone receptor coactivators

The steroid receptor coactivator family

The steroid receptor coactivator (SRC) family is composed of three distinct but structurally and functionally related members: SRC-1 (nuclear receptor coactivator 1), SRC-2 (transcription intermediary factor 2/glucocorticoid receptor-interacting protein 1/nuclear receptor coactivator 2), and SRC-3 (p300/CREB-binding protein [CBP] cointegrator-associated protein/receptor-associated coactivator 3/activator of thyroid and retinoid receptors/amplified in breast cancer 1/thyroid receptor activator molecule 1). SRC-1 was the first identified coactivator for the steroid receptor superfamily, which was cloned and characterized in 1995 [10]. SRC-2 and SRC-3 were then identified thereafter by several laboratories [9].

Sequence analysis of SRC proteins identified a basic helix–loop–helix domain and two Per–Arnt–Sim domains in the amino-terminal region. The basic helix–loop–helix/

Per–Arnt–Sim domain is highly conserved among the SRC members, and it serves as a DNA binding and protein dimerization motif in many transcription factors [11]. Following the basic helix–loop–helix/Per–Arnt–Sim domain, there are a centrally located receptor-interacting domain and a C-terminal transcriptional activation domain. Detailed analysis revealed three conserved LXXLL motifs (nuclear receptor box) in the receptor-interacting domain, which appear to contribute to the specificity of coactivator–receptor interaction. Histone acetyltransferase activity was identified in the C-terminal region of SRC members, and there also exist activation domains that can interact with the CBP.

All three members of the SRC family interact with the PR and enhance its transcriptional activation in a ligand-dependent manner [12,13]. Targeted deletion of the SRC-1 gene in mice has indicated that SRC-1 is important for the biological actions of progesterone in mammary gland development since the hormone-induced ductal elongation and alveolar development is greatly impaired in the null mice [14]. In the meantime, the expression of SRC-2 mRNA was elevated in SRC-1 null mice, suggesting that SRC-2 can partially compensate for SRC-1 function [14].

SRC-3 is the most distinct among the three members. It coactivates not only the nuclear receptors, but also other unrelated transcription factors such as those in the cAMP or cytokine pathways [15]. Compared with the widespread expression of SRC-1 and SRC-2, expression of SRC-3 is restricted to the mammary gland and several other tissues [16]. Disruption of the SRC-3 gene in mice causes severe growth and reproductive defects, including the retardation of mammary gland development [17]. Furthermore, amplification and overexpression of SRC-3 were observed in 10% and 64% of human primary breast cancers, respectively [18]. This observation indicates that SRC-3 is not only essential for the normal mammary development, but also plays a role in breast tumorigenesis.

E6-associated protein/RPF1

E6-associated protein (E6-AP) and RPF1, the human homolog of yeast RSP5, are E3 ubiquitin-protein ligases that target proteins for degradation by the ubiquitin pathway. They are also characterized as coactivators of steroid receptors. It has been demonstrated by transient transfection assay that RPF1 and E6-AP could potentiate the ligand-dependent transcriptional activity of the PR, the glucocorticoid receptor, and other nuclear receptors [19,20]. Furthermore, RPF1 and E6-AP acted synergistically to enhance PR transactivation [20]. Additionally, the coactivation functions of E6-AP and RPF1 are separable from the E3 ubiquitin-protein ligase activity, as ubiquitin ligase-defective E6-AP and RPF1 exhibited normal coactivation function.

E6-AP is expressed in many tissues, including the mammary gland. From its ability to coactivate the PR and the ER in a hormone-dependent manner, it was assumed that E6-AP is an essential regulator for the development of normal mammary gland and mammary tumors. The first evidence of a relationship between E6-AP and breast cancers was obtained from the study of a spontaneous mouse mammary tumorigenesis model, which demonstrated that E6-AP was overexpressed in tumors when compared with normal tissues [21].

We recently examined the expression pattern of E6-AP in biopsy samples of human breast cancers, and our results showed that E6-AP expression was decreased in tumors in comparison with the adjacent normal tissues (Gao *et al.*, unpublished data, 2002). Furthermore, we demonstrated that the decreased expression of E6-AP was stage dependent, and that the expression of E6-AP was inversely correlated with that of the ER in breast tumors. Since the ER plays a major role in breast cancer development and the PR is a target of estrogen, the changes of the expression level of E6-AP might interfere with the normal functioning of the ER and the PR. Hence, E6-AP may participate in the formation and progression of breast tumors.

Steroid receptor RNA activator

The growing family of nuclear receptor coactivators has recently acquired a unique member, steroid receptor RNA activator (SRA) [22]. Differing from the other coactivators, SRA functions as a RNA transcript instead of as a protein. SRA specifically coactivates the transcriptional activity of steroid receptors, including the PR, the ER, the glucocorticoid receptor, and the androgen receptor. It has been demonstrated that SRA exists in a ribonucleoprotein complex containing SRC-1 and that it mediates transactivation through the AF-1 domain located at the N-terminal region of nuclear receptors, distinguishing it from the other coactivators.

SRA is expressed in normal and malignant human mammary tissues [23,24]. An elevated expression of SRA was found in tumors compared with the adjacent normal region [24]. Although it is currently unknown whether the expression of SRA is correlated with that of the PR or the ER, the increase in the SRA levels in tumor cells may contribute to the altered ER/PR action that is known to occur during breast tumorigenesis.

L7/SPA

L7/SPA is a 27 kDa protein containing a basic leucine zipper domain. L7/SPA is an antagonist-specific transcriptional coactivator because it can only potentiate the partial agonist activity of some antagonists, including tamoxifen and RU486, but has no effect on the agonist-mediated transcription [25]. This unique property of L7/SPA suggests that it might play a role in the development of resistance to hormone therapy for breast cancers.

CBP/p300

CBP was initially characterized as a coactivator required for efficient transactivation of cAMP-response element-binding protein, and p300 was first identified as a coactivator of the adenovirus E1A oncoprotein. CBP and p300 share many functional properties: both of them function as coactivators for multiple nuclear receptors as well as p53 and nuclear factor-κB [9], both possess intrinsic histone acetyltransferase activity, and both can recruit histone acetyltransferase and CBP/p300-associated factor [26]. Besides, CBP/p300 interacts with members of the SRC family and synergizes with SRC-1 in the transactivation of the ER and the PR [27].

Other coactivators

In addition to the coactivators already discussed, there are a few other proteins that have been demonstrated to upregulate the transcriptional activity of the PR. Chromatin high-mobility group protein 1, chromatin high-mobility group protein 2, TIP60 (Tat-interacting protein), proline-rich nuclear receptor coregulatory protein 1, proline-rich nuclear receptor coregulatory protein 2, Cdc25B, and GT198 all function as PR coactivators, as demonstrated by transient transfection assays [28–32]. Cdc25B is prominent among these coactivators in terms of its roles in breast cancer development, because Cdc25B transgenic mice exhibit mammary gland hyperplasia and increased steroid hormone responsiveness [31]. The significance of all these coactivators *in vivo* needs to be further investigated.

Progesterone receptor corepressors

Nuclear receptor corepressor/silencing mediator of retinoid and thyroid receptors

Nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid receptor (SMRT) are both corepressors of numerous transcription factors, including steroid hormone receptors. Both N-CoR and SMRT interact with the nuclear receptors through the receptor-interacting domains located in the C-terminal portion of the proteins, while their transcriptional repression domains are mapped to the N-termini [33]. N-CoR and SMRT also associate with HDAC3 in large protein complexes, which is an important pathway for transcriptional repression. Corepressors N-CoR and SMRT interact with the nuclear receptors either in the absence of agonists (in the case of the thyroid receptor and the retinoid acid receptor) or in the presence of antagonists (in the case of steroid receptors) [33]. Since N-CoR and SMRT are common corepressors for transcription factors, slight alteration of their expression level in certain tissues might result in significant transcriptional changes, leading to altered development of the mammary gland, even tumors.

BRCA1

BRCA1 is a breast cancer susceptibility gene, and its inherited mutations are correlated with an increased risk of breast cancer and ovarian cancer [34]. The role of *BRCA1* in cancer development is unclear. In addition to its ability to coactivate p53 and to modulate p300/CBP expression, *BRCA1* is also a ligand-independent corepressor for the ER, the androgen receptor and the PR [35]. If *BRCA1* is mutated, all of these pathways will be more or less impaired. The effect of *BRCA1* in cancer development might therefore be multiplex.

Other corepressors

Ubiquitin-activating enzyme 3 (Uba3) is the catalytic subunit of the activating enzyme in the ubiquitin-like NEDD8 (neural precursor cell-expressed developmentally downregulated) conjugation (neddylation) pathway. Uba3 was recently demonstrated as a corepressor of the ER, the androgen receptor and the PR in mammalian transfection assays [36]. Uba3 inhibited the transactivation of the ER in a time-dependent manner, and neddylation activity of Uba3 is required for this suppression. This suggests that Uba3 suppresses steroid receptor activity by promoting the termination of receptor-mediated gene transcription rather than by interfering with the initial events.

Repressor of tamoxifen transcriptional activity (RTA) has recently been defined as a potent repressor of tamoxifen-mediated ERα transcriptional activity as well as an agonist of the ERβ, the glucocorticoid receptor, and the PR [37]. The interaction of RTA with the nuclear receptors requires the participation of RNA, because mutation of the RNA recognition motif in RTA compromises its ability to repress transcription [37].

The roles of Uba3 and RTA in mammary gland development and tumorigenesis await further study.

Summary

As a transcription factor, the PR activates target gene transcription in response to the hormonal stimulus, and its functions are modulated by coactivators and corepressors. Different coregulators exert their actions through different mechanisms, and involvement in the development of normal mammary gland and the formation or progression of tumors has been reported in some coactivators and some corepressors. The coactivators and corepressors of the PR so far identified are not PR specific, since they can also modulate the transactivation of many other nuclear receptors. In addition, no unique coregulators of PR-A or PR-B have been identified. Identification of PR-specific coregulatory proteins, especially PR-A interacting factors or PR-B interacting factors, is an important goal of future study.

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